Functional and molecular characteristics of a helminth immunomodulator-induced suppressive macrophage population

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1 ZUSAMMENFASSUNG

Eine Eigenschaft chronischer Helmintheninfektionen ist die lange Verweildauer der Parasiten für Jahre oder Jahrzehnte im infizierten Wirtsorganismus. Um solche Lebensspannen zu erreichen, wirken Helminthen der Immunantwort ihrer Wirte durch Evasionsmechanismen entgegen, zum Beispiel durch die Sekretion immunmodulierender Moleküle. In diesem Zusammenhang wurde in Vorarbeiten gezeigt, dass Filarien-Cystatin (AvCystatin) Parameter allergischer Atemwegsentzündung in Mäusen in einer IL-10 abhängigen Weise signifikant reduzieren konnte (Schnoeller et al., 2008). Die Beobachtung, dass eine Depletion von Makrophagen diese Immunsuppression wieder aufhob, führte zu der Hypothese, dass ein Makrophagen-vermittelter Mechanismus für die Linderung von Krankheitssymptomen verantwortlich sein muss. Die vorliegende Arbeit hatte zum Ziel, AvCystatin-modulierte Makrophagen (AvCystatin-MΦ) in zwei wesentlichen Ansatzpunkten zu analysieren. Erstens wurden Signalwege ermittelt, die in peritonealen Makrophagen nach Behandlung mit dem Immunmodulator zur Produktion von IL-10 führten. Zweitens sollten die suppressorischen Eigenschaften von AvCystatin-MΦ und die molekularen Besonderheiten der Makrophagen-vermittelten Immunsuppression in zwei Krankheitsmodellen der Maus untersucht werden.

Die Stimulation von peritonealen Maus-Makrophagen mit AvCystatin führte zu einer frühen, jedoch transienten Expression von IL-10 in den Makrophagen und korrelierte mit einer Phosphorylierung der Mitogen-aktivierten Proteinkinasen (MAPK) p38 und ERK. Um die transiente IL-10-Expression in AvCystatin-MΦ zu verstehen, wurde der Einfluss von dual-spezifischen Phosphatasen (DUSPs) auf die MAPK-Aktivität und IL-10-Expression untersucht. Die Behandlung mit AvCystatin *in vitro* und *in vivo* führte zu einer Hochregulation der Phosphatasen DUSP-1, DUSP-2 und DUSP-5. Die erhöhte Expression von IL-10 und die Akkumulation von p38 in DUSP-1 defizienten Mäusen nach AvCystatin-Behandlung bekräftigte eine entscheidende Rolle der Phosphatase DUSP-1 für die Regulation der MAPK p38 und ERK und die damit verbundene IL-10-Expression. Diese Ergebnisse zeigen, dass AvCystatin Signalkaskaden in Makrophagen addressiert, um auf diese Weise die Produktion von IL-10 anzuregen.

Die funktionelle Charakterisierung von AvCystatin-MΦ in einem murinen Model der Ovalbumin-induzierten Atemwegsentzündung zeigte, dass suppressive Effekte unabhängig von der initialen IL-10-Expression der Makrophagen auf die inflammatorische Wirtsantwort vermittelt werden können. Bereits ein einmaliger Transfer von AvCystatin-MΦ führte zu einer prominenten Reduktion von Gesamt- und Allergen-spezifischem Serum-IgE, verminderte die Menge von Eosinophilen und Th2-Zytokinen in der Bronchoalveolarflüssigkeit, unterdrückte

die systemische Th2 Zytokin-Antwort und milderte die Schleimproduktion in den Atemwegen. Die AvCystatin-MΦ vermittelte Abschwächung entzündlicher Prozesse war mit signifikant erhöhten Mengen IL-10 in den Empfängertieren assoziiert. Um die Effekte von AvCystatin-MΦ in einem weiteren Krankheitsmodell zu untersuchen, wurden Makrophagen in Mäuse mit DSS-induzierter Kolitis transferiert. Die Auswertung von Kolonschnitten sowie die Analyse von inflammatorischen Zellen in der Submukosa des Kolons, Körpergewichtskontrollen und Messungen der Kolonlänge ergaben, dass die Behandlung mit AvCystatin-MΦ Mäusen einen effizienten Schutz gegen intestinale Entzündung vermitteln konnte.

In weiteren Untersuchungen wurde der Phänoptyp von *in vitro* und *in vivo* induzierten Makrophagen untersucht. Quantitative Real-time PCR von AvCystatin-MΦ zeigte in beiden Fällen eine frühe und transiente Expression der pro- und anti-inflammatorischen Markergene iNOS, IL-12/23p40, TNF-α, IL-6 und IL-10. Dieser Phänotyp wechselte nach 18-20 Stunden zu einer M2a/M2b Hybrid-Signatur, charakterisiert durch die Expression der Markergene Arginase-1 (ARG-1), Sphingosinkinase-1 (SPHK-1) und LIGHT (TNFSF14). Eine durchflusszytometrische Analyse von Oberflächenmarkern der $F4/80^+CD11b^+$ AvCystatin-MΦ zeigte eine Hochregulation von MHC-II, kostimulatorischen Molekülen (CD40, CD80 und CD86), Fcγ-Rezeptoren (CD16/32), PD-L1 (CD274), PD-L2 (CD 273) und ICAM-1.

Um den zugrunde liegenden Mechanismus der AvCystatin-MΦ vermittelten Induktion von IL-10 in T-Zellen besser zu verstehen, wurde eine *in vitro* Kokultur aus Makrophagen, dendritischen Zellen und Ovalbumin-spezifischen $CD4^+$ T-Zellen etabliert. Dabei zeigte sich, dass AvCystatin-MΦ die IL-2-, IFN-γ- und IL-13-Sekretion von $CD4^+$ T-Zellen unterdrücken, gleichzeitig aber einen signifikanten Anstieg von IL-10 in diesen T-Zellen bewirken. Die Generierung von $CD4^+IL\text{-}10^+$ T-Zellen erfolgte zellkontaktunabhängig und war spezifisch für den *in vivo* induzierten Phänotyp der AvCystatin-MΦ. Eine Blockade des IL-10 Rezeptors auf T-Zellen vor Kokultivierung mit dendritischen Zellen und Makrophagen führte zu keiner Umkehrung der durch AvCystatin-MΦ vermittelten Effekte.

Zusammenfassend konnte in der vorliegenden Arbeit eine durch das immunmodulierende Molekül AvCystatin induzierte suppressive Makrophagenpopulation charakterisiert werden, die unabhängig von der Anwesenheit des Immunomodulators gegen Entzündungsreaktionen in verschiedenen Krankeitszusammenhängen schützt.

2 ABSTRACT

A feature of chronic helminth infections is the long parasite persistence for years or decades in the infected vertebrate host. To achieve such life spans, helminths counteract host immune responses with a panel of evasion mechanisms, e.g. through the secretion of immunomodulatory molecules. In this context, previous studies reported on filarial cystatin (AvCystatin) which reduced clinical parameters of allergic airway inflammation in mice in an IL-10-dependent manner (Schnoeller et al., 2008). Since depletion of macrophages reversed the observed immunosuppression, a macrophage-mediated mechanism was hypothesized to be responsible for the amelioration of disease. The present study aimed to analyse AvCystatin-stimulated macrophages (AvCystatin-MΦ) in two major steps. First, signalling events were investigated which led to IL-10 producion in macrophages after treatment with the immunomodulator. Second, the efficiency and molecular characteristics of AvCystatin-MΦ-induced immunosuppression was determined in two murine disease models.

Stimulation of murine peritoneal macrophages with AvCystatin induced an early but transient IL-10 expression in macrophages and correlated with phosphorylation of mitogen-activated protein kinases (MAPK) p38 and ERK. To understand the transient nature of IL-10 expression in AvCystatin-MΦ, the impact of dual-specificity phosphatases (DUSPs) on MAPK activity and IL-10 expression was examined. Treatment of macrophages with AvCystatin *in vitro* and *in vivo* induced up-regulation of DUSP-1, DUSP-2 and DUSP-5. Increased IL-10 expression and the accumulation of p38 in DUSP-1-deficient mice after treatment with AvCystatin confirmed a predominant role of DUSP-1 for the regulation of p38 and ERK and the associated IL-10 expression. These findings show that AvCystatin targets signalling pathways in macrophages to induce IL-10 production.

Functional characterization of AvCystatin-MΦ in a murine model of ovalbumin-induced airway inflammation showed that AvCystatin-MΦ can mediate suppression on inflammatory host responses independent of the initial IL-10 expression by the macrophages. Single application of AvCystatin-MΦ led to a prominent reduction of total and allergen-specifc serum IgE, reduced the amount of eosinophils and Th2 cytokines in the bronchoalveolar lavage fluid, suppressed systemic Th2 cytokine responses and mitigated mucus production in the airways. The resolution of allergic inflammation was associated with significantly increased levels of IL-10 in recipient mice. To asses the effects of AvCystatin-MΦ in an additional disease model, AvCystatin-MΦ were transferred into mice suffering from DSS-induced colitis. Intravenous treatment with AvCystatin-MΦ efficiently protected mice against the development of intestinal inflammation as determined by histological analysis of colon sections, evaluation

of inflammatory cell influx into the submucosal compartment, control of body weight and measurement of colon length.

In subsequent studies the phenotype of *in vitro*- and *in vivo*-induced peritoneal AvCystatin-MΦ was analysed. Quantitative real-time PCR in both cases revealed an early and transient expression of pro- and anti-inflammatory marker genes such as iNOS, IL-12/23p40, TNF-α, IL-6 and IL-10 after contact with the immunomodulator. This phenotype changed towards a suppressive M2a/M2b hybrid signature after 18-20 hours which was characterized through the expression of arginase-1 (ARG-1), sphingosine kinase-1 (SPHK-1) and LIGHT (TNFSF14). Surface marker analysis of the $F4/80^+CD11b^+$ AvCystatin-MΦ through flow cytometry showed an up-regulation of MHC-II, co-stimulatory molecules (CD40, CD80 and CD86), Fcγ receptors (CD16/32), PD-L1 (CD274), PD-L2 (CD273) and ICAM-1.

To understand the underlying mechanism of IL-10 induction by AvCystatin-MΦ, an *in vitro* co-culture assay was established in which macrophages were incubated with dendritic cells and ovalbumin-specific $CD4^+$ T cells. AvCystatin-MΦ suppressed the secretion of IL-2, IFN-γ and IL-13 but induced significant amounts of IL-10 in $CD4^+$ T cells. The induction of $CD4^+IL\text{-}10^+$ T cells was cell contact independent and specific for *in vivo*-stimulated AvCystatin-MΦ. Blockade of the IL-10 receptor on $CD4^+$ T cells prior to co-culture with dendritic cells and macrophages did not reverse the effects of AvCystatin-MΦ.

In summary, the present thesis characterized the molecular characteristics of a suppressive macrophage population induced by the helminth immunomodulator AvCystatin which transfers protection against unrelated inflammation in different disease contexts independent of the immunomodulator`s presence.

3 INTRODUCTION

Helminths are multicellular organisms which live as extracellular parasites at the expense of other organisms. They obtain nourishment from and reproduce in their hosts thereby inflicting morbidity. Beyond that, helminth parasites enhance susceptibility to viruses, bacteria and protozoan parasites (Hotez et al., 2008) and thus constitute a big health problem. To date, approximately one quarter of the world population is infected with helminth parasites. However, while helminth infections are rare in the western world, they are highly prevalent in developing and tropical countries. Helminths are usually divided into nematodes (roundworms), trematodes (flukes) and cestodes (tapeworms). Among these, nematodes are especially abundant. Parasitic nematodes are transmitted through arthropod vectors, faeco-orally or actively penetrate the vertebrate host. Prominent gastrointestinal nematodes are *Trichuris trichiura* and hookworms such as *Ancylostoma duodenale* or *Necator americanus*. Important tissue-dwelling nematodes are represented by *Brugia malayi*, *Wuchereria bancrofti*, *Onchocerca volvulus* and *Loa loa*. Although nematodes can cause pathology (e.g. river blindness, elephantiasis or sowda), many chronically infected patients remain asymptomatic (van Riet et al., 2007). This means that helminth infections do not necessarily have to be accompanied by severe illness but rather suggests that well-adapted parasites prevent impetuous immune responses by tolerizing the host immune system and thereby avert damage to the parasitized host.

3.1 Helminth infections and the modulation of immune disorders

In recent years, epidemiological and laboratory studies confirmed that helminth parasites modulate the host immune system to ensure their survival and thereby exert spillover effects on unrelated immune dysfunctions such as atopy. This shows that besides many negative aspects of helminth infections, parasites also exhibit characteristics which can probably be exploited to treat aberrant immune reactions. Atopic diseases encompass a wide range of immunologic disorders. Among these, allergic asthma is a frequently occurring disease in westernized countries. It affects 200 to 300 million people worldwide (Braman, 2006) and is defined as a type I hypersensitivity chronic inflammatory disorder of the airways triggered by innocuous antigens that are usually well tolerated. Several risk factors are considered to influence the susceptibility of asthma such as genetic background, age, gender, nutritional condition, immune status and environmental factors, e.g. air pollution or stress (reviewed in Subbaro et al., 2009). The course of disease is divided into two phases (Holt et al., 1999). During the induction phase, an inhaled environmental allergen gets internalized and processed by dendritic cells (**Figure 3-1, a**). Alternatively, lung epithelial cells secrete thymic

stromal lymphopoietin (TSLP) and directly equip dendritic cells with Th2-promoting capacity (**Figure 3-1, b**). The interaction between dendritic cells and $CD4^+$ T cells leads to the development of Th2 effector T cells (**Figure 3-1, c**) which are key players for the establishment of Th2 immune responses. They secrete cytokines that favour disease induction and progression, namely IL-4, IL-5, IL-9 and IL-13. IL-5 induces recruitment and maturation of eosinophils (**Figure 3-1, d**). IL-4 promotes mast cell maturation (**Figure 3-1, e**) and, in combination with IL-13, triggers both isotype switching in B cells and the secretion of allergen-specific IgE by plasma cells (**Figure 3-1, f**). The secretion of IL-9 supports IL-5 in promoting eosinophilopoiesis and acts synergistically with IL-4 on mast cell development. In addition to Th2 cytokines, production of the stem cell factor (SCF) by epithelial cells supports mast cell recruitment (**Figure 3-1, g**). A number of activated $CD4^+$ Th2 effector T cells develop into Th2 memory T cells which remain in a quiescent stage until they are activated through re-exposure to the same antigen (**Figure 3-1, h**). During the effector phase of asthma, the antigen binds to FcεRI-attached allergen-specific IgE on mast cells, basophils and eosinophils in a bivalent manner (**Figure 3-1, i**) which triggers the release of bioactive mediators (e.g. histamine, leukotrienes, prostaglandins etc.) from intracellular granules (**Figure 3-1, j**). Such factors cause smooth muscle contraction, increased bronchial mucus production and stimulation of sensory nerves that finally lead to clinical manifestation of asthma which is characterized through cardinal symptoms like itching, wheezing, coughing, airway obstruction and dyspnoea.

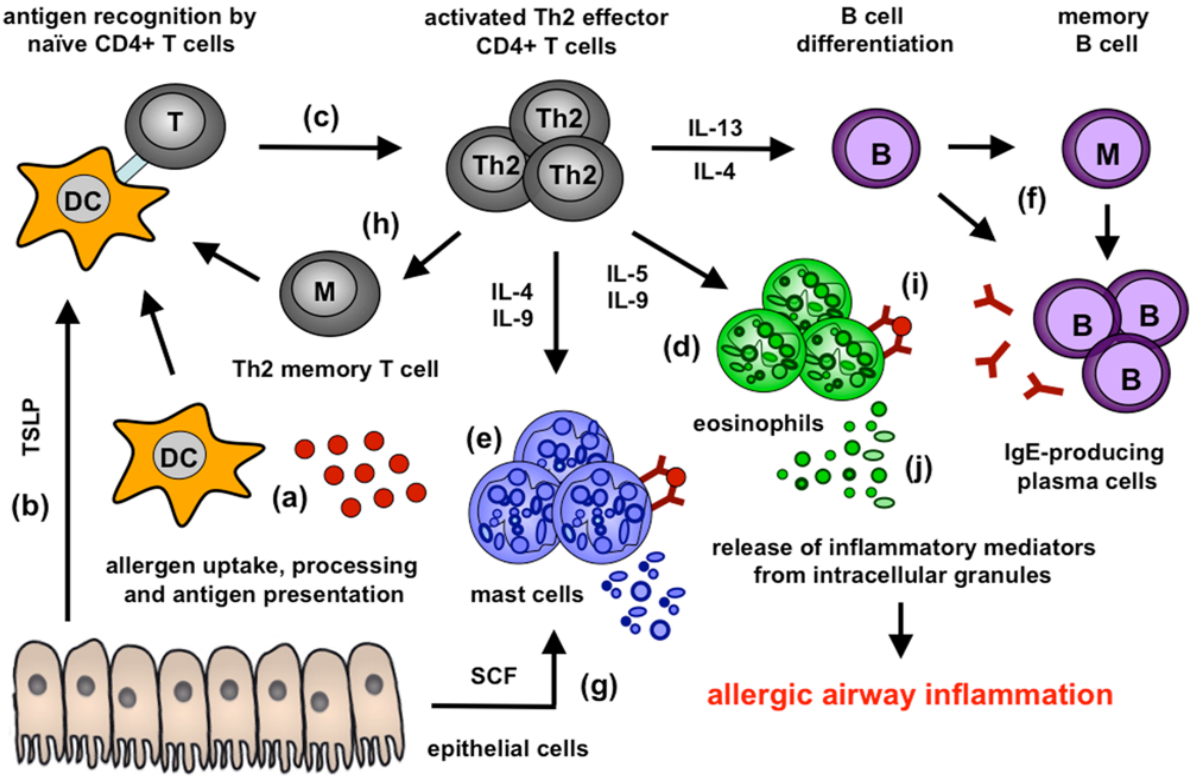

Figure 3-1. Development of allergic airway inflammation. Allergic airway inflammation is induced through activation of dendritic cells by antigen (a) or TSLP (b). Interaction of dendritic cells with naïve $CD4^+$ T cells evoke their differentiation into $CD4^+$ Th2 effector T cells (c) that produce specific cytokines. Secreted IL-4, IL-5, IL-13 and IL-9 lead to eosinophilia (d), mast cell recruitment (e) and production of antigen-specific IgE by plasma cells (f). Secretion of SCF by epithelial cells further supports the recruitment and activation of mast cells (g). Moreover, Th2 memory cells are generated which remain in a quiescent state until there are re-activated through antigen-specific contact (h). Antigen binding to FcεRI-attached IgE on mast cells or eosinophils (i) leads to the release of mediators (j) which cause inflammation and pathophysiological modifications.

In industrialized countries the prevalence of asthma and other allergy-related diseases has been remarkably rising in the last decades (ISAAC, 1998; Cooper, 2009). Formerly, this trend was explained by the “hygiene hypothesis” saying that an exposure to microbial infections during early childhood shifts the balance of Th1 and Th2 immune responses towards an anti-allergic Th1 response that counterbalance pro-allergic Th2 immunity and prevents the tendency to develop allergies (Strachan, 1989). Hence, reduced exposure to Th1-polarizing bacterial or viral infections in the western world as a result of improved public health, high hygiene standards and the early use of antibiotics or vaccinations could have given rise to an increased susceptibility to inappropriate immune responses to allergens. Two important observations, however, raised doubts against the model of Th1/Th2 counter regulation proposed by the hygiene hypothesis. First, not only did the incidence of allergic diseases rise dramatically in westernized countries, also Th1-biased autoimmune diseases, e.g. multiple sclerosis, type 1 diabetes or morbus crohn, considerably increased during the last decades (Bach, 2002). Second, helminth parasites induce Th2-phenotypic responses

which are reminiscent to immunological responses in allergic disorders. Helminths could thus be expected to promote atopic disorders (Yazdanbakhsh et al., 2001), however, epidemiological studies found atopy to be less frequent in areas where helminth infections are endemic (Yazdanbakhsh et al., 2001; Nyan et al., 2001, Scrivener et al., 2001; Cooper et al., 2003; Dagoye et al., 2003). This inverse correlation raised a considerable interest in the question whether helminths ameliorate rather than exacerbate allergic diseases. Further laboratory studies verified protective effects of helminth infections in animal models of allergic inflammation and, by assuming that helminth immunosuppression might be a nonspecific process, addressed whether parasitic worms could also affect other dysregulated immune responses. Meanwhile beneficial effects of helminth parasites or secreted helminth molecules have successfully been evaluated in murine disease models for type 1 diabetes (Cooke et al., 1999; Saunders et al., 2007), arthritis (McInnes et al., 2003), experimental autoimmune encephalomyelitis (La Flamme et al., 2003) and colitis (Elliott et al., 2004; Smith et al., 2007). Furthermore, successful treatment of human inflammatory bowel disease patients with *Trichuris suis* or *Necator americanus* in clinical trials demonstrated that helminths can be used for therapeutic purposes (Summers et al. 2005; Croese et al., 2006). In consequence, a lively interest has emerged in particular in parasite-elicited immunoregulatory mechanisms.

3.2 Immunoregulation by helminth parasites

Helminths are powerful immunoregulators that evade and modulate the immune system of their hosts (Maizels et al., 2004; Hewitson et al., 2009). Being in close contact with tissue, parasitic worms are directly exposed to the host`s immune response. The longevity of these pathogens for decades or even life-long in immunocompetent hosts, however, illustrates that they have shaped effective strategies to ensure their survival.

3.2.1 Strategies of helminth immune evasion

Helminth immune evasion includes a large repertoire of strategies. One such strategy is the migration through tissues and compartments in the host`s body or to dwell and hide in nodules. Moreover, parasitic worms are able to mask their surface with host-like molecules (molecular mimikry) or avoid antibody opsonisation and subsequent activation of the host`s immune system by either the secretion of antibody-degrading proteases or, mechanically, by moving and molting thereby peeling off the opsonising antibodies. Helminth parasites further modulate the host immune response towards a phenotype that has been designated as a “modified Th2 immune response” (Platts-Mills et al, 2001 a). Such immunomodulation is an important aspect of immune evasion and often associated with the induction of immunoregulatory cells that mediate cellular hyporesponsiveness. Regulatory T cells, for example, control innate and adaptive immune responses. They suppress immune reactions to allergens, prevent pro-inflammatory cytokine production by dendritic cells, convey suppressive activity to macrophages, inhibit proliferation of effector T cells and dampen both Th2 and Th1 cell responses (Hawrylowicz and O´Garra, 2005; McKee and Pearce, 2004; Kryczek et al., 2006; Grainger et al., 2010). Similarly, specific subpopulations of helminth-derived B cells are described to exhibit suppressive functions either directly by the secretion of IL-10 or through the induction of $Foxp3^+$ regulatory T cells (Mangan et al., 2004; Amu et al., 2010). In addition, antigen-presenting cells (APC) are important targets of helminth-secreted products. The modulation of dendritic cell functions by helminths was shown to affect dendritic cell cytokine responses (Massacand et al., 2009), to influence the expression of co-stimulatory surface molecules on dendritic cells (Segura et al., 2007) and to support the induction of IL-10-producing regulatory T cells (van der Kleij et al., 2002). Helminth-induced macrophages show context-dependent functions. They are described as important mediators of tissue remodelling and either suppress T effector cells thereby promoting parasite survival or mediate host protection against parasite (re)infection.

The secretion of anti-inflammatory cytokines such as transforming growth factor β (TGF-β) or IL-10 by worm-induced regulatory cells is a key feature of helminth immunomodulation. TGF-β has been shown to suppress cells of the innate immune system, to induce peripheral tolerance and to maintain the survival of regulatory T cells (Li et al., 2006). IL-10 was originally identified to suppress cytokine production by Th1 cells (Fiorentino et al., 1989) and therefore termed “cytokine synthesis inhibitory factor”. Meanwhile, it is known that IL-10 has multiple effects on different cell types. It down-regulates the activity of NK cells (Couper et al., 2008) and inhibits mast cell cytokine production, degranulation and IgE receptor expression (Arock et al., 1996; Royer et al., 2001; Norton et al., 2008). IL-10 further suppresses APC functions through the inhibition of major histocompatibility complex class II (MHC-II) or co-stimulatory molecule expression (de Waal Malefyt et al., 1991; Fiorentino et al., 1991) which, in turn, affects the development of Th1 and Th2 effector responses. In addition, IL-10 influences the balance of antibody isotypes by suppressing class switching to antigen-specific IgE and promoting the induction of IgG4 (Jeannin et al., 1998). While antigen-specific IgE is protective and involved in pathology, the IgG4 isotype suppresses complement activation (van der Zee et al., 1986) and counteracts IgE by competing for allergen binding sites thereby preventing IgE-mediated effector cell activation and degranulation. Such an uncoupling of specific IgG4 and IgE has been defined as a main feature of a modified Th2 response (Maizels and Yazdanbakhsh, 2003). Similar to IgG4, increased levels of parasite-induced polyclonal IgE have been considered to saturate IgE binding sites on effector cells which prevent degranulation through binding of allergen-specific IgE (Holt et al., 1999; Erb, 2007). Since this so-called “IgE blocking” or “mast cell saturation hypothesis” had been postulated, it was used as a feasible explanation for the observation that anaphylactic reactions often occur in atopics but never or rarely in helminth-infected individuals (Erb, 2007). The hypothesis was supported by early field studies showing that suppressive effects on atopy in helminth infections were associated with increased levels of polyclonal IgE (Godfrey, 1975; Merrett et al., 1976; Lynch et al., 1993). Additional findings, however, challenge the IgE-blocking theory, for example, by showing that *Nippostrongylus brasiliensis*-induced polyclonal IgE is able to block passive sensitization in rats but not hypersensitivity reactions mediated by actively produced IgE (Jarrett et al., 1980).

In brief, helminth immune evasion has emerged as an essential prerequisite for parasite survival and propagation. In particular the ability to actively modulate host immunity is suggested to be most likely effected through the secretion of mediators which target and modify host immune cells in manifold ways (Hewitson et al., 2009).

3.2.2 Helminth immunomodulators

The secretion of bioactive molecules is a hallmark of helminth immunomodulation. Once released, such mediators target host cells, interfere with signalling pathways and modify effector cell functions. Over the past few years, a couple of secreted factors have been identified and evaluated as important immunomodulators.

Aminopeptidases. The leucine aminopeptidase ES-62 is the predominant ES product of the filarial nematode *Acanthocheilonema viteae* (Harnett et al., 2004). ES-62 is one of the best characterized immunomodulators and exerts its biological activity on a wide range of host immune cells through modifications of phosphorylcholine (Houston and Harnett, 2004; reviewed in Hewitson et al., 2009). ES-62 suppresses $CD4^+$ T cell proliferation and T cell-mediated secretion of IFN-γ and IL-4 (Marshall et al., 2005), promotes IL-10 production by B1 cells (Wilson et al., 2003) and affects B cell-T cell interactions (Marshall et al., 2008). ES-62 was further shown to inhibit IL-12 production by dendritic cells and macrophages in response to lipopolysaccharide (LPS) (Goodridge et al., 2001 and 2003), prevented FcεRI-mediated mast cell degranulation in a model of ovalbumin-induced airway inflammation (Melendez et al., 2007) and protected mice against collagen-induced arthritis (McInnes et al., 2003). These findings show that ES-62 is a potent immunomodulatory molecule that holds great promise for therapeutical application.

Cytokine homologues. Parasite-secreted cytokine homologues mimick biological functions of mammalian host cytokines. *Brugia malayi* homologues of the macrophage migration inhibitory factor (MIF) was shown to modify the activity of human monocytes and macrophages (Pastrana et al., 1998) and synergized with IL-4 for the induction of alternatively activated macrophages through up-regulation of the IL-4 receptor (Prieto-Lafuente et al. 2009). TGF-β homologue 2 (TGH-2) of *Brugia malayi* was found to bind the mammalian TGF-β receptor and supposed to promote the induction of $Foxp3^+$ regulatory T cells (McSorley et al., 2008). *De novo* expression of Foxp3 in T cells via the TGF-β pathway has been described for a TGF-β mimick of the gastrointestinal nematode *Heligmosomoides polygyrus* called *Heligmosomoides polygyrus* excretory-secretory antigen or HES (Grainger et al., 2010). Similarly, MIF homologues of *Anisakis simplex* (As-MIF) suppressed clinical parameters of Th2 inflammation in a murine model of allergic airway inflammation through the recruitment of $Foxp3^+$ regulatory T cells (Park et al., 2009).

C-type lectins and galectins. Parasite-derived C-type lectins were found to mimick different host molecules. TES-32 and TES-70 of the dog roundworm *Toxocara canis* show high homology to mammalian proteins like the low affinity receptor for IgE (CD23) or the mannose receptor (CD206) on macrophages. TES-70 was further determined to mask worm carbohydrates by binding mammalian carbohydrates (Loukas et al. 1999 and 2000; reviewed in Hewitson et al., 2009). Galectins are carbohydrates that have been discussed as "amplifiers, silencers or tuners of inflammatory responses" (Rabinovich et al., 2002). Galectins of *Haemonchus contortus* are capable of modulating eosinophil migration *in vitro* (Turner et al., 2008) and *Onchocerca volvulus* beta galactoside binding protein (OvGalBP) was found to bind IgE thus protecting the parasite from eosinophil-mediated damage (Klion and Donelson, 1994; reviewed in Johnston et al., 2009).

Lipid molecules, glycanes and glycoproteins. Lysophosphatidylserine (lyso-PS) is a schistosomal lipid molecule that acts on dendritic cells through the activation of TLR-2 and thereby induces IL-10-producing regulatory T cells (van der Kleij et al., 2002). Lacto-N-neotetraose (LNnT) and lacto-N-fucopentaose III (LNFPIII) are important glycans of the blood fluke *Schistosoma mansoni*. Intraperitoneal injection of LNnT-dextran (LNnT-dex) into mice was shown to induce a $Gr1^+CD11b^+F4/80^+$ cell population which produces low levels of pro-inflammatory cytokines but high amounts of IL-10 and TGF-β and suppresses T cell proliferation *in vitro* (Terrazas et al., 2001). LNFPIII was determined to promote APC maturation through the activation of NF-kappa B (Thomas et al., 2005) and to induce alternative activation of macrophages (Atochina et al., 2008). Symilarly, glycans of *Taenia crassiceps* metacestodes were found to induce alternatively activated macrophages through up-regulation of ARG-1, Ym1 and CD206 in murine peritoneal macrophages (Gómez-García et al., 2006). In 2003, a dimeric glycoprotein was purified from soluble egg antigen (SEA) of *Schistosoma mansoni* and named IL-4-inducing principle of *Schistosoma mansoni* eggs (IPSE) due to its ability to induce IL-4 in human basophils (Schramm et al., 2003). IPSE was shown to be identical with a formerly described antigen in SEA called alpha-1 (Dunne et al., 1981) and since then designated as IPSE/alpha-1 (Schramm et al., 2006). IPSE/alpha-1 was determined to induce secretion of IL-4 and IL-13 by basophils thereby supporting alternative activation of macrophages.

Protease inhibitors. Protease inhibitors such as cystatins constitute effective modulatory molecules which are widely spread and involved in various biological processes. They display a group of evolutionary related tight-binding but reversible inhibitors of papain-like cysteine proteases (Nicklin and Barrett, 1984). Parasite-derived cystatins interact with both parasite and host proteases and have been described for several parasite species (reviewed

in Klotz et al., 2011 a). They regulate endogenous physiological processes such as moulting or oogenesis, protect the parasite against host proteases, interfere with antigen processing/presentation thereby affecting T cell priming and further modulate host cytokine responses or nitric oxide (NO) production (reviewed in Klotz et al., 2011 a). Two systems are currently used to classify cystatins. The older system differentiates three major types of cystatins (Barrett, 1986). First, stefins (type 1 cystatins) which are unglycosylated and intracellularly located proteins. They have a single domain but lack a signal sequence and disulfide bonds. Second, cystatins (type 2 cystatins) that possess a signal sequence for extracellular targeting but no carbohydrate side chains. They have a single domain with two conserved disulfide bonds at the carboxy terminus, are secreted and thus detectable in almost all biological fluids. Finally, kininogens (type 3 cystatins) are glycosylated proteins which are synthesized in the liver and exported to the blood. They possess three type 2 cystatin-like domains with eight disulfide bonds. A newer classification system is provided by the MEROPS peptidase database (http://merops.sanger.ac.uk) that is based on amino acid sequences and structural characteristics of molecules. According to this classification, cystatins belong to the inhibitor family I25 that comprises inhibitors of family C1 (papain like) cysteine proteases and is subdivided into I25A, I25B and I25C.

The present thesis focused on AvCystatin, a cystatin C-like molecule (type 2 cystatin, I25B) secreted by all developmental stages of the tick-transmitted rodent filaria *Acanthocheilonema viteae* (Hartmann et al., 1997). *In vitro* stimulation of mouse splenocytes with recombinant AvCystatin suppressed $CD4^+$ T cell proliferation and induced IL-10 production (Hartmann et al., 1997). Additional studies revealed host cell modulation to be specific for AvCystatin since cystatin of the free-living nematode *Caenorhabditis elegans* did not exert regulatory effects (Schierack et al., 2003). Moreover, by evaluating *in vivo* functions of the immunomodulator, application of AvCystatin significantly reduced airway inflammation in a murine model of ovalbumin-induced airway hyperreactivity. These protective effects were reversed after depletion of macrophages suggesting that macrophages are targets of AvCystatin and exert important immunoregulatory functions (Schnoeller et al., 2008).

In summary, helminth parasites secrete factors which interfere with different arms of the host immune system and establish an environment which is beneficial for their survival and reproduction. In many cases, it has been shown that such immunomodulation can lead to spillover effects on unrelated inflammation. Hence, immune regulation by helminths can be advantageous for both parasite and host as it enables the maintenance of infection and parasite survival but also protects the host against immunopathology of unrelated disease.

3.3 Macrophage biology and function

The term “macrophage” has been attributed to Elie Metchnikoff who originally described these cells as large and efficient phagocytic cells (Metchnikoff, 1905). They originate from myeloid progenitor cells in the bone marrow which develop into monoblasts, promonocytes and monocytes. Monocytes leave the marrow and circulate in the blood until they enter tissues and differentiate into macrophages. Macrophages reside in virtually all organs throughout the body. They appear as microglia in the central nervous system, Langerhans cells in the skin, Kupffer cells in the liver, alveolar macrophages in the lungs, osteoclasts in bones and histiocytes in connective tissue. The complete range of cells including bone marrow progenitors, blood monocytes and macrophages constitutes a family of cells that has been defined as the mononuclear phagocyte system or MPS (Hume, 2006).

Besides their structural heterogeneity macrophages show high functional plasticity. Their primary role is to maintain homeostasis through cleaning the interstitial environment from cellular debris, erythrocytes and apoptotic cells (Mosser and Edwards, 2008). Another functional facet is their ability to mount immunological functions. Since macrophages are innate immune cells, they constitute the first line of defence and represent efficient detector cells for danger signals in early infection (Dunn et al., 1985). They recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs) such as mannose, scavenger, toll-like or complement receptors (reviewed in Plüddemann et al., 2007) and respond with phagocytic activity, antigen presentation and expression of co-stimulatory molecules for T cell activation. Moreover, macrophages migrate to the site of inflammation and act as effector cells through the release of bioactive mediators, cytokines and chemokines. The capacity of macrophages to produce anti-inflammatory cytokines highlights their role as regulatory cells, especially in cancer and parasitic infections (Reyes and Terrazas, 2007). Thus, macrophages are important cells that hold a variety of functions and thereby bridge innate and adaptive immune responses.

3.4 Polarization and classification of murine macrophages

To identify and distinguish macrophage subsets, studies have been focusing on how different stimuli influence the functional phenotype of macrophages. In 1992, Stein and collaborators described IL-4-activated macrophages to differ from IFN-γ-treated macrophages by increased mannose receptor expression and activity (Stein et al., 1992; reviewed in Gordon, 2003). In consequence, macrophages were divided along two axes into M1 and M2 macrophages to describe two main extremes of activation with distinct functions thereby mimicking the Th1/Th2 paradigm of activated T cells and their polarized cytokine profiles

(Mills et al., 2000). The term M1 describes classical activation of macrophages that arises due to injury or infection. M1 macrophages are induced by IFN-γ in combination with a second signal such as microbial products or tumor necrosis factor α (TNF-α). After activation, M1 macrophages produce pro-inflammatory cytokines (IL-12, IL-1β, TNF-α, IL-6 and IL-23), chemokines (CXCL-9, 10, 11 and 16), NO, reactive oxygen species (ROS) and show increased tumoricidal and phagocytic activity. M1 macrophages play an important role in polarized Th1 immune responses and mediate resistance against intracellular pathogens (Mantovani et al., 2005). M2, on the contrary, was introduced as a generic term that includes all forms of macrophage activation other than M1 thus representing "alternative activation" of macrophages. M2 cells are less efficient in killing pathogens but promote tissue repair and remodelling. Furthermore, by producing anti-inflammatory cytokines they suppress M1 macrophage activity and enhance anti-inflammatory responses (Martinez et al., 2008).

Mantovanti et al. (2004) expanded the original M1/M2 axis classification by dividing M2 macrophages into subpopulations according to their route of activation and the resulting phenotype (**Figure 3-2, A**). The most representative form of M2 polarization is M2a activation which is induced through stimulation with Th2 cytokines IL-4 and IL-13. Compared to M1 macrophages, M2a-polarized cells are less efficient APCs and fail to activate $CD4^+$ T cells or to induce $CD4^+$ T cell proliferation (Edwards et al., 2006). They are poor in producing NO but instead express high levels of ARG-1 which skews L-arginine metabolism towards proline and polyamine production (Hesse et al., 2001; reviewed in Stempin et al., 2009). Other marker genes expressed by M2a macrophages are CD206 (mannose receptor), resistin-like molecule α (RELM-α) and Ym1 (Raes et al., 2002). M2b macrophages constitute a physiologically distinct subpopulation that corresponds to a phenotype that has been described by Anderson and Mosser (2002) as type 2 macrophages. M2b cells are induced by immune complexes (IC) in the presence of TLR ligands. They produce high levels of IL-10, sphingosine kinase-1 (SPHK-1) and LIGHT (TNFSF14). However, M2b macrophages are not per se anti-inflammatory. They still produce low amounts of IL-12, TNF-α, IL-1β, IL-6, present antigen to $CD4^+$ T cells and induce $CD4^+$ T cell proliferation (Edwards et al., 2006). M2c macrophages are elicited by IL-10, TGF-β or glucocorticoids. They down-regulate proinflammatory cytokines and can be regarded as deactivated macrophages (Martinez et al., 2008).

Macrophage polarization towards a functional phenotype is not irreversible. They retain the ability to respond to changes in their environment and adapt their phenotype by shifting from M1 to M2 activation or back (Stout et al., 2005; Mosser and Edwards, 2008). As macrophage activation is supposed to comprise a much wider spectrum than described in former

classification systems, Mosser and Edwards (2008) suggested a system based on three fundamental macrophage functions including host defense (classically activated macrophages), wound healing (wound healing macrophages) and immune regulation (regulatory macrophages) and illustrated these properties as the primary colours of a colour wheel (**Figure 3-2, B**). Since macrophages can show characteristics of different subtypes, this classification system describes macrophage heterogeneity more precisely by accounting for hybrid macrophages with overlapping transcription profiles analogous to secondary colours that arise through the combination of primary colours.

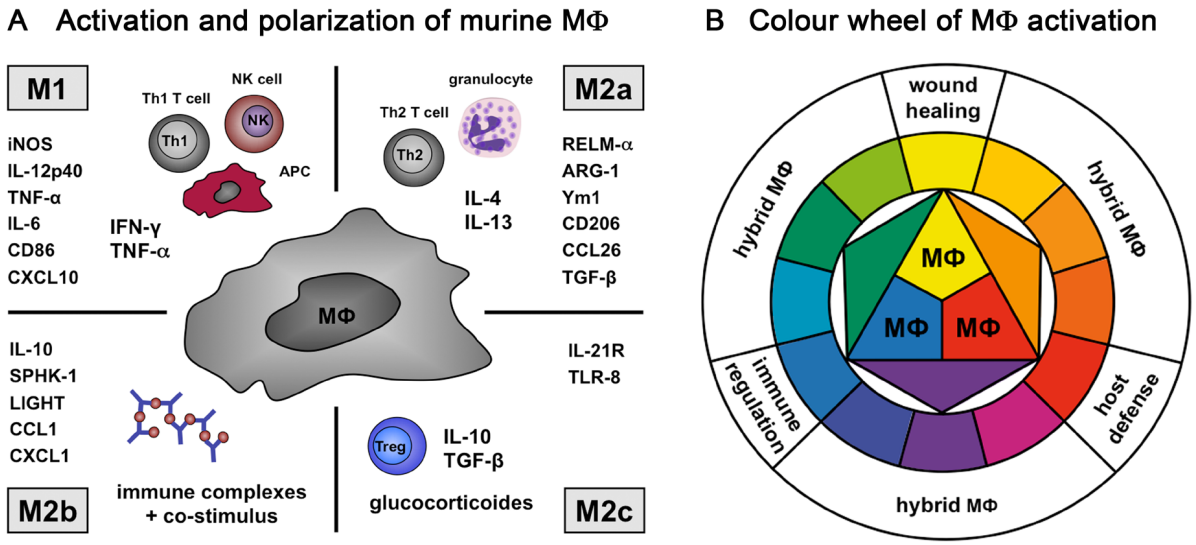

Figure 3-2. Activation and classification of murine macrophages. Macrophage classification includes a wide spectrum of physiologically distinct macrophages. (A) According to the activation stimuli and the resulting phenotype, macrophages are divided into classically activated M1 or alternatively activated M2 macrophages. Alternatively activated macrophages can be further differentiated into M2a, M2b and M2c macrophages (Martinez et al., 2008). (B) A newer classification system illustrates the three macrophage functions “wound healing”, “host defense” and “immune regulation” as the primary colours of a colour wheel. Similar to secondary colours that derive through the combination of primary colours, macrophages can share features of different phenotypes (Mosser and Edwards et al., 2008). The illustrated figures are based on figures in Martinez et al. (2008) and Mosser and Edwards (2008).

3.5 Macrophages and disease

Macrophages are critical for the initiation, maintenance or resolution of inflammatory processes (Fujiwara and Kobayashi, 2005) and thus show pathogenic but also protective activity in different disease settings.

3.5.1 Macrophages in allergic airway inflammation

Macrophages exhibit divergent functions in allergic responses and have long been assumed to promote or exacerbate allergic inflammatory processes in the airways of patients (Holgate, 2008; Verstraelen et al., 2008). On the contrary, there is experimental evidence which demonstrates the ability of macrophages to suppress allergic inflammation. The depletion of alveolar macrophages (AM), for instance, has been shown to increase IgE levels in mice suggesting a major role of macrophages in the regulation of IgE responses to inhaled allergen (Thepen et al., 1992). Similarly, Bang et al. (2011) found that depletion of alveolar macrophages increased the number of eosinophils, lymphocytes and levels of IL-4, IL-5 and GM-CSF in the bronchoalveolar lavage fluid (BAL-fluid) of mice suffering from asthma. Transfer of unsensitized alveolar macrophages, however, reduced eosinophil and lymphocyte numbers as well as GM-CSF concentrations in the BAL-fluid. Moreover, adoptive transfer of alveolar macrophages from allergy-resistant into alveolar macrophage-depleted and allergy-susceptible rats ameliorated airway hyperresponsiveness (AHR), substantiating the idea of a macrophage-mediated protection against AHR (Careau and Bissonette, 2004). Lung interstitial macrophages were found to prevent LPS-induced airway allergy by suppressing maturation and migration of dendritic cells (Bedoret et al., 2009). In another study, intratracheal application of immortalized splenic macrophages (Mf4/4 cells) pulsed *ex vivo* with ovalbumin successfully inhibited allergic airway responses in mice through the recruitment of IFN-γ-producing Th1 cells demonstrating that macrophages can suppress Th2-driven inflammation through the induction of counter-regulating Th1 responses (Pynaert et al., 2003). In summary, a rising number of studies show that macrophages are able to suppress airway inflammation and thus constitute important cells not only for the induction but also for negative regulation of allergic responses.

3.5.2 Macrophage functions in autoimmunity

Experimental data account for protective macrophage functions in a number of autoimmune diseases. *In vivo* application of immune complexes to ligate Fcγ receptors in mice protected animals against experimental autoimmune encephalomyelitis (EAE) (La Flamme et al., 2006). As immune complexes are involved in the generation of regulatory type 2 (M2b)

macrophages, La Flamme and colleagues examined the influence of *in vitro*-generated and intraperitoneally injected M2b or M1 macrophages on EAE in mice. Animals treated with M2b macrophages showed less severe signs of EAE whereas the administration of M1 macrophages had no effect on EAE development (Tierney et al. 2009). In another context, murine macrophages successfully protected against T cell-dependent arthritis by secreting ROS which suppressed T cell proliferation (Gelderman et al., 2007). Moreover, expression of RANTES on ocular-infiltrating macrophages abrogated clinical parameters in a murine model of experimental autoimmune uveoretinitis (EAU) whereas neutralization of RANTES impaired EAU by changing ocular-infiltrating T cell subsets (Sonoda et al., 2003). The peroxisome proliferator-activated receptor-γ (PPARγ) has been correlated with alternative activation of murine macrophages in obesity (Heilbronn and Campbell, 2008) and is supposed to control immune responses (reviewed in Nencioni et al., 2003). Shah et al. (2007) elucidated a protective role of PPARγ in a model of dextran sodium sulfate (DSS)-induced colitis by showing that mice with PPARγ-null macrophages but not wildtype animals developed aggravated symptoms of intestinal inflammation. Thus, macrophage suppressive activity is not restricted to atopic disorders but can also protect against diverse forms of autoimmunity.

3.5.3 Tumor-associated macrophages (TAM)

In cancer, macrophages exert tumoricidal functions or contribute to tumor growth and disease progression. Since M1 macrophages are known to perform tumoricidal activity it comes as no surprise that tumor cells promote pro-tumoral macrophage activation by driving an M2-like phenotype of human and murine macrophages (Biswas and Mantovani, 2010). Tumor-associated macrophages (TAM) are ineffective in presenting antigens and characterized by low expression levels of NO and pro-inflammatory cytokines IL-12, IL-6, TNF-α and IL-1β (Mantovani et al., 2002). On the contrary, they produce high amounts of IL-10 and TGF-β (Sica et al., 2002), express CD206 (Allavena et al., 2010), scavenger receptor A (Biswas et al., 2006) and promote angiogenesis (Sica et al., 2002). Neovascularisation is important for tumor progression and has been associated with TAM-mediated release of proangiogenic factors, e.g. vascular endothelial growth factor or thymidine phosphorylase (Hotchkiss et al., 2003). Tumor-derived agents that drive M2 macrophage polarization are not fully determined yet but depletion of TAM has been shown to successfully reduce tumor progression and angiogenesis (De Palma et al., 2005; Zeisberger et al., 2006) suggesting that macrophage targeting might be of great importance to control tumor growth (Solinas et al., 2009).

3.6 Macrophage functions in helminth infections

Infections with parasitic worms are usually associated with macrophages that show high phenotypic similarity to those exposed to Th2 cytokines IL-4 and IL-13 (M2a activation). Such macrophages have increasingly become an active field of scientific interest and research (Jenkins and Allen, 2010). Literature research on murine macrophages in helminth infections shows that they exert diverse but sometimes also contradicting biological functions depending on the infection stage, infection site and the respective parasite species (Jenkins and Allen, 2010).

3.6.1 Regulation of Th2 responses by helminth-induced macrophages

Helminth-induced macrophages are important regulators of Th2 immune responses. They either support the recruitment of Th2 effector cells thereby mediating parasite clearance and host protection (positive regulation) or suppress $CD4^+$ T cell functions and promote parasite survival (negative regulation).

3.6.1.1 Positive regulation of Th2 responses and host protection

A couple of studies suggested that helminth-induced macrophages promote the development of $CD4^+$ Th2 cells and contribute to the recruitment of Th2 effector cells such as eosinophils (Hayashi et al., 1999; Mills et al., 2000). It was shown, that the induction of alternatively activated macrophages during *Nippostrongylus brasiliensis* infection correlated with the recruitment of eosinophils to the peritoneal cavity of mice whereas markedly reduced eosinophils numbers were detected in infected macrophage-deficient *op/op* mice (a mouse strain with impaired development of mononuclear phagocytes due to an inactivating mutation in the colony-stimulating factor 1 gene) and macrophage-depleted animals (Voehringer et al., 2007). These results were supported by studies that describe alternatively activated macrophages to participate in the recruitment of eosinophils to the lungs of mice in a model of ovalbumin-induced airway inflammation (Ford et al., 2009; Ford et al., 2012) and by the finding that eosinophils appear to direct development of alternatively activated macrophages in intestinal helminth infection models (Loke et al., 2007; Byers and Holtzmann, 2010).

Macrophage function in host protection was first reported by investigating early events in the memory response to larvae of the gastrointestinal parasite *Heligmosomoides polygyrus*. After primary infection, Th2 memory cells prevented parasite re-infection by inducing alternatively activated macrophages in an IL-4- and STAT6-dependent manner. Blockade of ARG-1 reversed the effects and led to recovery of larval stages. Thus, ARG-1 emerged to be an

important effector molecule in mediating host protection against helminth re-infection (Anthony et al., 2006). Similar findings were obtained for intestinal macrophages of mice infected with *Nippostrongylus brasiliensis*. Both the depletion of macrophages by clodronate liposomes and the blockade of ARG-1 in macrophages reduced intestinal smooth muscle hypercontractility suggesting a macrophage-dependent and again, ARG-1-mediated mechanism to be responsible for parasite clearance (Zhao et al., 2008). *Hymenolepis diminuta* infection was associated with the expression of ARG-1, FIZZ-1 (RELM-α) and Ym1 in the intestine of mice as early as 8 days post infection suggesting the induction of alternatively activated macrophages. Since the appearance of these macrophages correlated with the peak of worm expulsion, the authors hypothesized the macrophages to mediate protection against worm infection (Persaud et al., 2007).

3.6.1.2 Negative regulation of Th2 responses and parasite survival

Contrariwise, recent data illustrate that helminth-modulated macrophages also act as negative regulators of Th2 immune responses thereby protecting the parasite from clearance rather than inducing worm expulsion. *In vivo* depletion of *Taenia crassiceps*-induced macrophages drastically reduced worm burdens in infected mice and favoured resistance to *Taenia crassiceps* (Reyes et al., 2010). STAT6-deficient but not wildtype mice were shown to develop strong Th1 immune responses associated with high levels of IFN-γ, IL-12 and NO post infection with *Taenia crassiceps* cysticerci. Thus, STAT6 signalling-dependent expansion of macrophages is essential for mediating susceptibility to *Taenia crassiceps* (Rodríguez-Sosa et al., 2002 a and b).

The suppression of effector cell functions is an important mechanism to negatively regulate Th2 responses. First evidence for a suppressive activity of helminth-derived macrophages was found when mice were intraperitoneally implanted with adult *Brugia malayi* or injected with *Brugia malayi* ES products. Peritoneal macrophages were *in vitro* co-cultured with polyclonal or antigen-specific stimulated $CD4^+$ T cells and successfully suppressed T cell proliferation in a cell contact and IL-4-dependent manner (Loke et al., 2000; MacDonald et al., 1998). Similar activity was found for macrophages induced by ES products from the rodent hookworm *Nippostrongylus brasiliensis* and the dog roundworm *Toxocara canis* (Allen and MacDonald, 1998). Moreover, experimental infection with *Litomosoides sigmodontis* induced ARG-1-expressing $F4/80^+$ macrophages in mice which suppressed antigen-specific $CD4^+$ T cell proliferation in an IL-10- and CTLA-4-independent but partially TGF-β-dependent manner (Taylor et al., 2006). Successional studies verified macrophages to exert suppressive activity on T cells in trematode and cestode infection models as well. In a murine

model of *Schistosoma mansoni* infection, splenic macrophages induced $CD4^+$ T cell anergy *in vitro* in a cell contact-dependent manner through the up-regulation of programmed death ligand 1 (PD-L1) (Smith et al., 2004). *Taenia crassiceps*-elicited peritoneal macrophages similarly prevented $CD4^+$ T cell proliferation through the up-regulation of both PD-L1 and PD-L2 (Terrazas et al., 2005). In a study by Mejri and Gottstein (2006), peritoneal macrophages of *Echinococcus multilocularis*-infected mice were stimulated with ovalbumin and suppressed the activity of *in vitro* co-cultured $CD4^+$ T cells of ovalbumin immunized mice. Together, these findings identify T cells as an important target of helminth-induced macrophages in order to suppress Th2 responses.

In recent studies, two molecules in particular were described as critical regulators of Th2 immunity: RELM-α and ARG-1. RELM-α is a cysteine-rich protein which was originally detected in the BAL-fluid of mice suffering from allergic airway inflammation and thought to modulate the local tissue response to allergic inflammation (Holcomb et al., 2000). However, experimental findings of Nair et al. (2009) demonstrated RELM-α expression in macrophages to inhibit pulmonary inflammation in a murine model of *Schistosoma mansoni* infection. In the respective study, RELM-α was shown to bind $CD4^+$ Th2 cell effector cells thereby suppressing cytokine production in a Bruton`s tyrosine kinase-dependent manner. Macrophages from RELM-α-deficient mice, on the other side, induced massive airway inflammation after challenge with *Schistosoma mansoni* eggs. Since RELM-α also bound macrophages and dendritic cells, the protein was suggested to exert other, non T cell-mediated functions as well (reviewed in Jenkins and Allen, 2010). Studies by another group showed that RELM-α-deficient mice developed stronger Th2 responses and increased worm expulsion following *Nippostrongylus brasiliensis* infection in comparison to infected wildtype mice. Moreover, *Schistosoma mansoni* egg-induced granuloma formation in the airways of mice was aggravated in the absence of RELM-α and associated with high serum IgE and increased eosinophil numbers. The investigators thus hypothesized RELM-α to act as an important feedback mechanism to suppress helminth-induced Th2 responses (Pesce et al., 2009 a). Similarly, ARG-1 has been described to exert suppressive activity on Th2 inflammation in chronic schistosomiasis. Conditional deletion of ARG-1 in macrophages of *Schistosoma mansoni*-infected mice led to increased liver fibrosis, portal hypertension and granulomatous inflammation. In addition, IL-4/IL-13-induced but not ARG-1-deleted macrophages were able to suppress $CD4^+$ T cell proliferation *in vitro* (Pesce et al., 2009 b) substantiating a critical role of ARG-1 in the suppression of Th2 inflammation.

3.6.2 Wound healing

An important feature of helminth-derived macrophages is their ability to orchestrate wound healing. At the site of injury, they remove dead cells, recruit fibroblasts, dissolve fibrin and further regulate angiogenesis and remodelling of connective tissue (Gordon, 2003). Tissue-dwelling worms are large metazoan organisms that release proteolytic enzymes to pass through tissues thereby causing cellular damage and organ injury accompanied by inflammatory host responses (Kreider et al., 2007). Tissue repair by macrophages thus exhibits an effective strategy of parasites to minimize tissue injury and to prevent inflammation. In a study by Herbert et al. (2004), macrophage-mediated wound repair was found to be critical for host survival during infection with *Schistosoma mansoni*. Compared to infected control animals, IL-4Rα-deficient mice were inefficient in inducing tissue remodelling macrophages and therefore died from excessive intestinal inflammation caused by egg penetration trough the intestine. The expression of ARG-1, which converts L-arginine to proline for collagen production, plays a key role in tissue remodelling (Albina et al., 1990). However, other markers might also contribute to this function. RELM-α was found to promote vascularization through the induction of angiogenic factors and thereby could promote fibroblast recruitment, differentiation and activation (Chung et al., 2007; Nair et al., 2009). Another candidate is Ym1, a secretory 45 kDa heparin-binding lectin that shows sequence similarities to chitinases (Hung et al., 2002). Although lacking chitinase activity, Ym1 can effectively bind to chitin or the extracellular matrix and thereby mediate the recruitment of cells which support tissue remodelling (Reece et al., 2006). In a study by Kzhyshkowska et al. (2006), the expression of scavenger-like receptor stabilin-1 on IL-4-stimulated murine macrophages led to binding and uptake of secreted protein acidic and rich in cysteine (SPARC), a matricellular glycoprotein which, among other functions, exerts anti-adhesive and anti-proliferative effects on diverse cell types. Removal of SPARC from the extracellular space by alternatively activated macrophages was suggested to regulate tissue remodelling and the synthesis of extracellular matrix components by cells which respond to SPARC (Kzhyshkowska et al., 2006).

In conclusion, helminth-derived macrophages bear diverse and sometimes opposing activities. They promote host Th2 immune responses and worm expulsion or suppress Th2 effector mechanisms and mediate parasite survival. Such functional discrepancy, however, might simply reflect the diversity of helminth infections and the complexity of the parasite-induced Th2 immune response (Jenkins and Allen, 2010). Thus, a single well-defined function for helminth-induced macrophages is unlikely to appear (Jenkins and Allen, 2010).

3.7 Aims of the thesis

The finding that helminth-secreted molecules interfere with unrelated inflammation suggests the possibility to use such factors for the treatment of immune-related diseases. Former studies showed that *in vivo* application of the immunomodulator AvCystatin significantly suppressed important parameters of ovalbumin-induced airway hyperreactivity in mice such as eosinophilia, allergen-specific IgE and mucus production (Schnoeller et al., 2008). The protective effects were associated with increased levels of IL-10 and reversed either through the depletion of macrophages or blockade of the IL-10 receptor. Thus, macrophages were hypothesized to be critically involved in the amelioration of disease possibly through the secretion of IL-10 (Schnoeller et al., 2008). The present study aimed (a) to pinpoint the molecular mechanism for the induction of suppressive macrophages by AvCystatin and (b) to further evaluate the ability of AvCystatin-modulated macrophages (AvCystatin-MΦ) to transfer protective effects in unrelated disease settings. The first part of the present investigation determined signalling events in macrophages which led to IL-10 production after treatment with AvCystatin. In the second part, the protective potential of AvCystatin-MΦ was examined by adoptive transfer experiments using murine disease models of allergic airway and intestinal inflammation. To gain further insights into the underlying mechanism of immunosuppression, transcriptional profiling of AvCystatin-MΦ was performed by quantitative real-time PCR and the expression of surface marker molecules analysed by flow cytometry.

4 RESULTS

4.1 Macrophages are target cells of AvCystatin

In order to identify the primary target cell of AvCystatin, the fate of DyLight549-labelled AvCystatin was analysed after application to the peritoneal cavity of BALB/c mice. Peritoneal exudate cells (PEC) were isolated 20 minutes after intraperitoneal administration of the protein, stained for surface marker molecules of specific cell populations (CD4, CD19, CD11c, CD11b and F4/80) and analysed by flow cytometry. AvCystatin was detected in $F4/80^{+}CD11b^{+}$ macrophages and $CD11c^{+}F4/80^{-}$ dendritic cells (**Figure 4-1, A**). Macrophages accounted for 35% and dendritic cells for 1.5% of the total PEC population suggesting resident macrophages to constitute the predominant target cells of AvCystatin within the peritoneal cavity (data not shown). AvCystatin was not determined in $Gr1^{high}$ cells excluding infiltrating monocytes or granulocytes as target cells (data not shown). Neither $CD4^{+}$ T cells nor $CD19^{+}$ B cells showed binding or uptake of the helminth immunomodulator (**Figure 4-1, A**). Confocal microscopy further determined AvCystatin to be taken up into the endolysosomal compartment of $CD11b^{+}$ phagocytes as revealed by the punctuate signal of the labelled molecule (**Figure 4-1, B**). To differentiate between specific binding and active uptake, PEC were incubated with labelled AvCystatin for 30 minutes at either 4°C or 37°C. Flow cytometry analysis revealed an uptake of AvCystatin by $F4/80^{+}$ cells at 37°C but minimally at 4°C. Incubation of PEC with labelled AvCystatin in the presence of actin polymerization-blocking cytochalasin D reduced protein uptake by macrophages (**Figure 4-1, C**). Furthermore, the kinetics of AvCystatin uptake by macrophages was determined by western blot analyses which showed a rapid and prolonged uptake between 0 and 24 hours at 37°C. Incubation at 4°C or 37°C in the presence of cytochalasin D for 15-60 minutes, however, reduced the uptake of AvCystatin (**Figure 4-1, D**). In conclusion, these findings confirm macrophages as the primary target cells of AvCystatin within the PEC population and propose an active endocytotic uptake of the protein but no or little binding to the cell surface of macrophages.

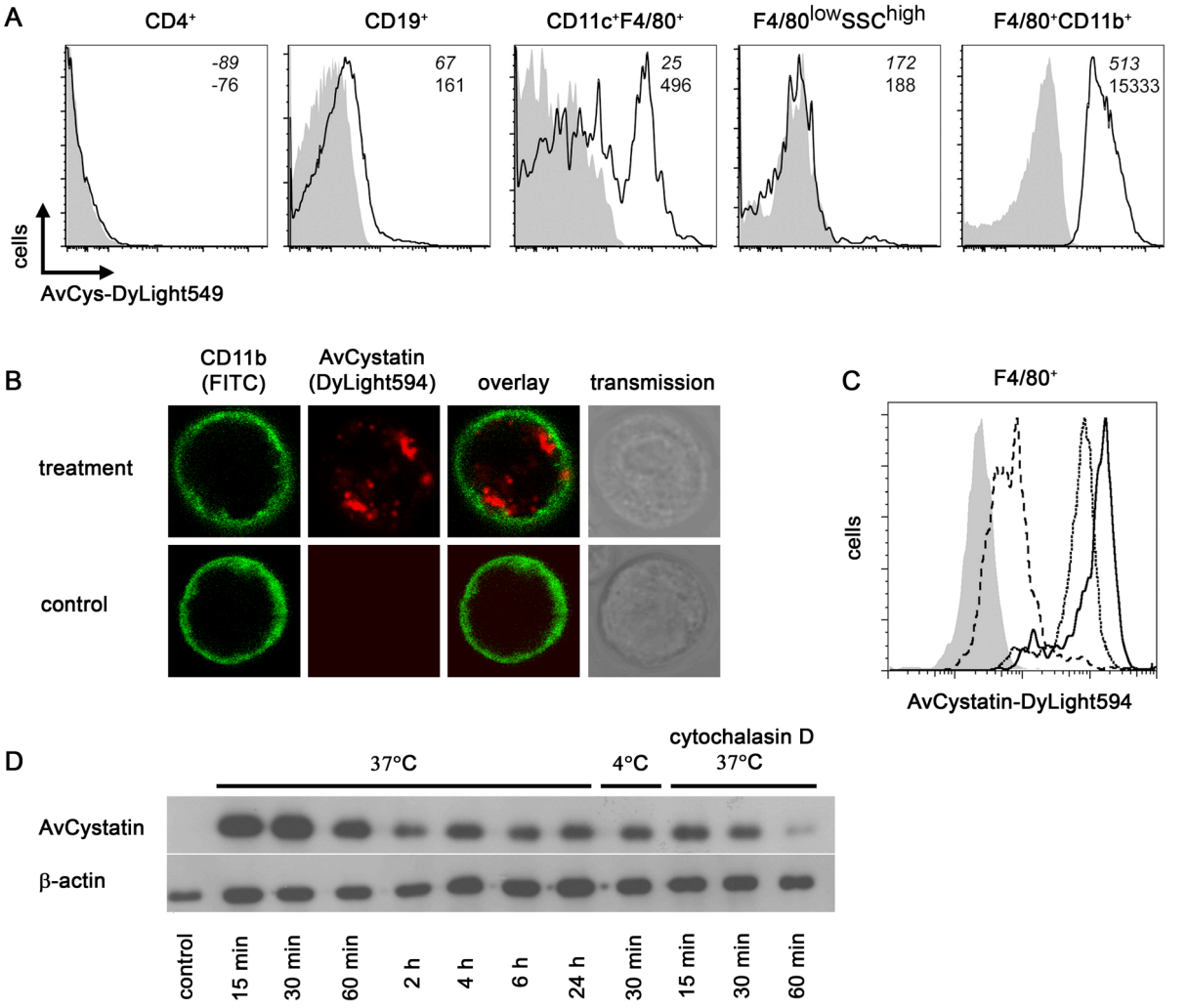

Figure 4-1. AvCystatin is actively taken up by peritoneal macrophages. (A) AvCystatin was labelled with DyLight549 and intraperitoneally injected into mice (40 µg/mouse). Peritoneal exudate cells were isolated from untreated (grey shaded area) and labelled AvCystatin-treated (white area) animals 20 minutes post application, stained with antibodies against CD4, CD19, CD11c, CD11b and F4/80 and analysed by flow cytometry. Numbers represent the mean fluorescence intensity. (B) Confocal microscopy of $CD11b^+$ cells (green) from the peritoneum of mice treated with DyLight594-labelled AvCystatin (red, upper panel) or untreated control mice (lower panel). (C) PEC were *in vitro* incubated with DyLight549-labelled AvCystatin for 30 min at either 37°C (solid line) or 4°C (long dashes). PEC were in addition treated with 10 µM cytochalasin D in combination with DyLight-AvCystatin for 30 minutes at 37°C (dotted line). Cells were stained against F4/80 and analysed for DyLight549 signals by flow cytometry. The shaded area represents untreated control cells. (D) Western blot analysis of cell extracts from AvCystatin-treated PEC at 37°C and 4°C. Some samples were additionally pre-treated with cytochalasin D to block endocytosis. Data are representative of 2-3 experiments.

4.2 Part I: Signalling events in AvCystatin-MΦ

IL-10 has been described as an important mediator in allergy and asthma (Hawrylowicz and O`Garra, 2005). Since previous studies demonstrated the importance of macrophages and IL-10 to mediate AvCystatin effects *in vivo*, the induction of immunosuppressive macrophages by AvCystatin was hypothesized (Schnoeller et al., 2008). The first part of the present thesis investigated molecular mechanisms of IL-10 induction in macrophages by dissecting signalling events leading to cytokine production after treatment with the immunomodulator.

4.2.1 MAPK activation by AvCystatin is associated with IL-10 production

The activation of mitogen-activated protein kinases (MAPK) ERK and p38 has been shown to correlate with IL-10 production in M2b macrophages (Lucas et al., 2005). In order to analyse signalling pathways involved in the AvCystatin-induced IL-10 production by macrophages, murine peritoneal macrophages were cultured in the presence of specific inhibitors for the MAPK extracellular signal-regulated kinase (ERK), p38, C-Jun N-terminal kinase (JNK) and the phosphatidylinositol-3-kinase (PI3K)-AKT pathway. First, the induction of IL-10 in macrophages was validated after incubation with AvCystatin or control treatments (denatured AvCystatin or the unrelated control protein SNAP-tag which constitutes a 20 kDa mutant of the DNA repair protein O6-alkylguanine-DNA alkyltransferase). IL-10 was found to be significantly increased in culture supernatants of AvCystatin- but not control-stimulated macrophages 18-20 hours after treatment (**Figure 4-2, A**). Real-time PCR analysis of IL-10 transcript revealed an early and transient expression of IL-10 in AvCystatin-MΦ that peaked one hour after treatment and decreased afterwards to background levels (**Figure 4-2, B**). Inhibition of PI3K activation through treatment with different concentrations of the inhibitor Ly294002 prevented IL-10 production in macrophages in a dose-dependent manner (**Figure 4-2, C**). Similarly, blockade of p38 and ERK through treatment with the inhibitors SB203580 (inhibition of p38) and U0126 (inhibition of MEK1/2) markely reduced levels of IL-10 in the culture supernatants (**Figure 4-2, D** and **E**). Incubation of macrophages with JNK-inhibitor II, however, had no effect on IL-10 production (**Figure 4-2, F**). To investigate the influence of tyrosine kinases on IL-10 production in macrophages, peritoneal macrophages were incubated with AvCystatin in the presence of the tyrosine kinase inhibitor genistein. Treatment with the inhibitor led to a dose dependent reduction of macrophage-secreted IL-10 (**Figure 4-2, G**). In conclusion, AvCystatin-mediated induction of IL-10 in macrophages involves p38, ERK and PI3K-dependent signalling events in a tyrosine kinase-sensitive manner.

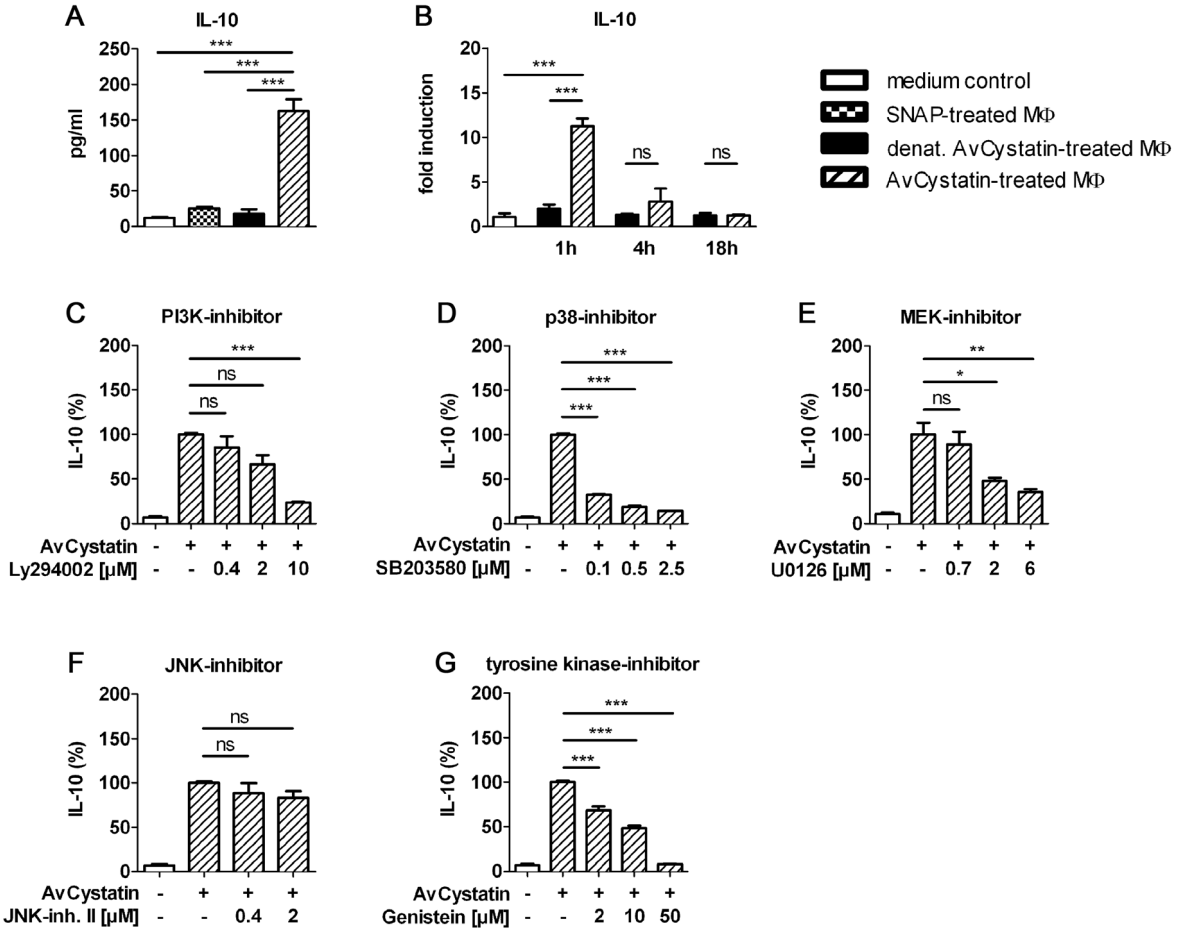

Figure 4-2. Induction of IL-10 involves ERK-, p38- and PI3K-dependent signalling. Induction of IL-10 in murine peritoneal macrophages after *in vitro* stimulation with AvCystatin for 18-20 hours in the absence or presence of specific inhibitors. (A) IL-10 production by macrophages after treatment with AvCystatin or control applications for 18 hours. (B) Real-time PCR analysis of IL-10 transcript in macrophages after incubation with AvCystatin or control treatment for 1, 4 and 18 hours. Normalized data is presented as fold induction compared to untreated macrophages (medium control). (C-G) Incubation of macrophages with AvCystatin and different concentrations of Ly294002 (PI3K-inhibitor), SB203580 (p38-inhibitor), U0126 (MEK1/2-inhibitor), JNK inhibitor II (JNK-inhibitor) and genistein (tyrosine kinase-inhibitor). One representative of 2-4 experiments is shown (A-G) and data (mean ± SEM) presented as percent cytokine production compared to AvCystatin treatment (C-G). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4.2.2 AvCystatin leads to phosphorylation of MAPK p38 and ERK

Next, the phosphorylation of ERK, p38 and AKT was examined by western blot analysis of cell lysates from AvCystatin- or control-treated peritoneal macrophages. Phosphorylation of p38 was determined by 15 minutes after application of AvCystatin, peaked at 60 minutes and decreased between 60 and 360 minutes (**Figure 4-3, A**). The ERK phosphorylation signal emerged as early as 15 minutes after treatment with the immunomodulator, remained elevated between 30 and 120 minutes and decreased from 120 minutes onwards (**Figure 4-3, B**). Although inhibition of PI3K affected IL-10 production in macrophages (**Figure 4-2, C**), no specific phosphorylation of AKT by AvCystatin was detectable (**Figure 4-3, C**). These findings show that treatment with AvCystatin induces transient phosphorylation of p38 and ERK in macrophages but does not affect phosphorylation of AKT.

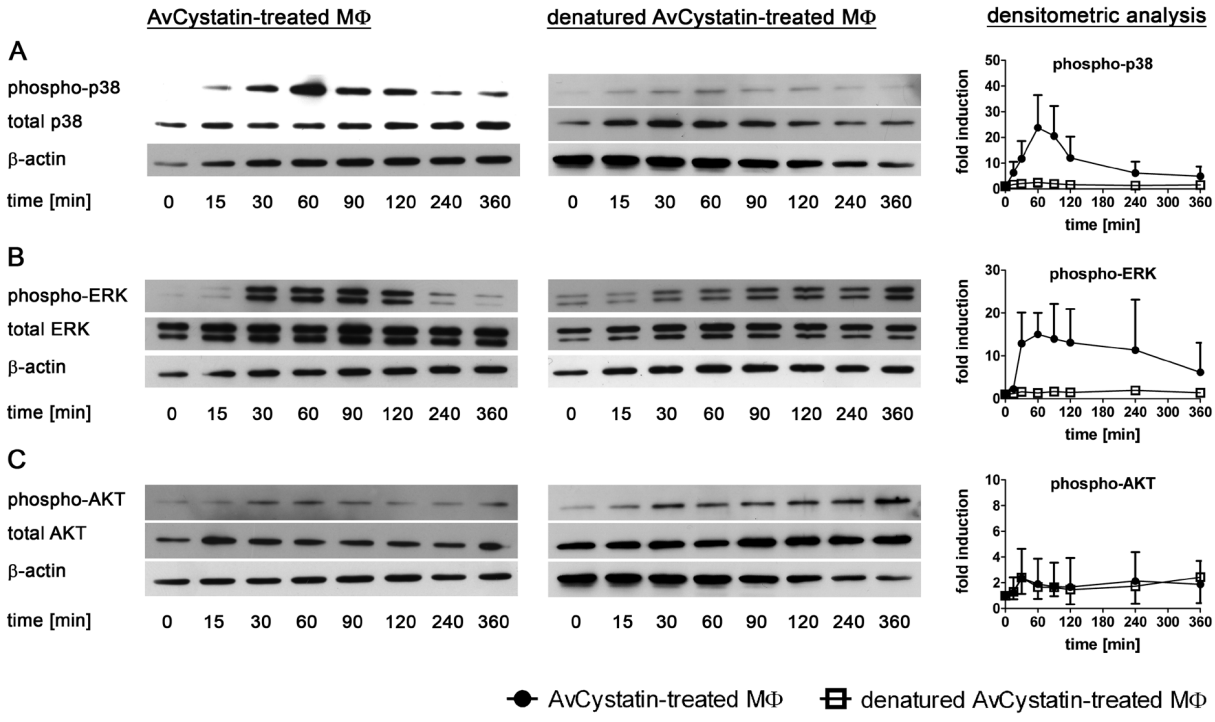

Figure 4-3. AvCystatin activates MAPK in peritoneal macrophages. Peritoneal macrophages were stimulated *in vitro* with 0.5 µM AvCystatin or denatured AvCystatin for various time points. Total cell extracts were analysed by western blotting of phospho- and total-p38, phospho- and total-ERK, phospho- and total-AKT. (A) Representative blot of phosho- and total p38 from AvCystatin- or control-treated cell extracts and densitometric blot analysis. (B) Blot of phospho- and total-ERK and densitometric analysis of protein expression. (C) Blot of phospho- and total AKT and densitometric analysis. Densitometric analysis data from one of 2-3 experiments is presented as mean ± SEM and expressed as fold induction relative to medium control. Phospho-p38, phospho-ERK and phospho-AKT were normalized to the endogenous control β-actin.

4.2.3 AvCystatin induces *in vitro* and *in vivo* expression of DUSPs

Since treatment with AvCystatin induced transient IL-10 expression and MAPK activation in macrophages, the involvement of a negative feedback mechanism on MAPK was hypothesized. Dual-specificity phosphatases (DUSPs) constitute a group of protein tyrosine phosphatases which dephosphorylate both tyrosine and threonine residues on MAPK thereby acting as negative regulators of MAPK activity (Jeffrey et al., 2007; Liu et al., 2007). In order to assess whether DUSPs influence the macrophage IL-10 response to AvCystatin, quantitative real-time PCR was performed for early expression of DUSPs which regulate p38 and ERK activity in macrophages (DUSP-1, DUSP-2, DUSP-3, DUSP-5, DUSP-6 and DUSP-10). *In vitro* stimulation of peritoneal macrophages with 0.5 µM AvCystatin induced an up-regulation of DUSP-1, DUSP-2 and DUSP-5 (**Figure 4-4, A**). Increased transcript levels were detected as early as 30 minutes post treatment for all three phosphatases. Expression of DUSP-1 reached its peak at 60 minutes and dropped between 60 and 240 minutes. DUSP-2 transcript levels were constant for indicated timepoints. A lower expression of DUSP-5 was detectable between 30 and 120 minutes after treatment with AvCystatin. Transcript levels of DUSP-3, DUSP-4 and DUSP-10 RNA remained unaltered (**Figure 4-4, A**). Next, DUSP-1 expression and phosphorylation was examined in total cell extracts by western blot analysis. Increased protein levels were detected by 60 and 90 minutes after treatment with AvCystatin. DUSP-1 phosphorylation was strongest after 60 minutes and again lowered after 90 minutes (**Figure 4-4, B**). To determine the *in vivo* effect of AvCystatin on DUSP expression, animals were intraperitoneally injected with 20 µg/mouse recombinant AvCystatin or denatured protein. Real-time PCR analyses of *ex vivo* purified peritoneal macrophages showed transient expression of DUSP-1 and DUSP-2 which both peaked 60 minutes after AvCystatin treatment and declined afterwards. DUSP-1 was found to be most prominently expressed whereas lower expression levels were detected for DUSP-2 (**Figure 4-4, C**). In summary, these findings show DUSPs to be expressed on RNA and protein level in AvCystatin-MΦ *in vitro* and *in vivo* suggesting that DUSPs regulate MAPK activity and IL-10 expression.

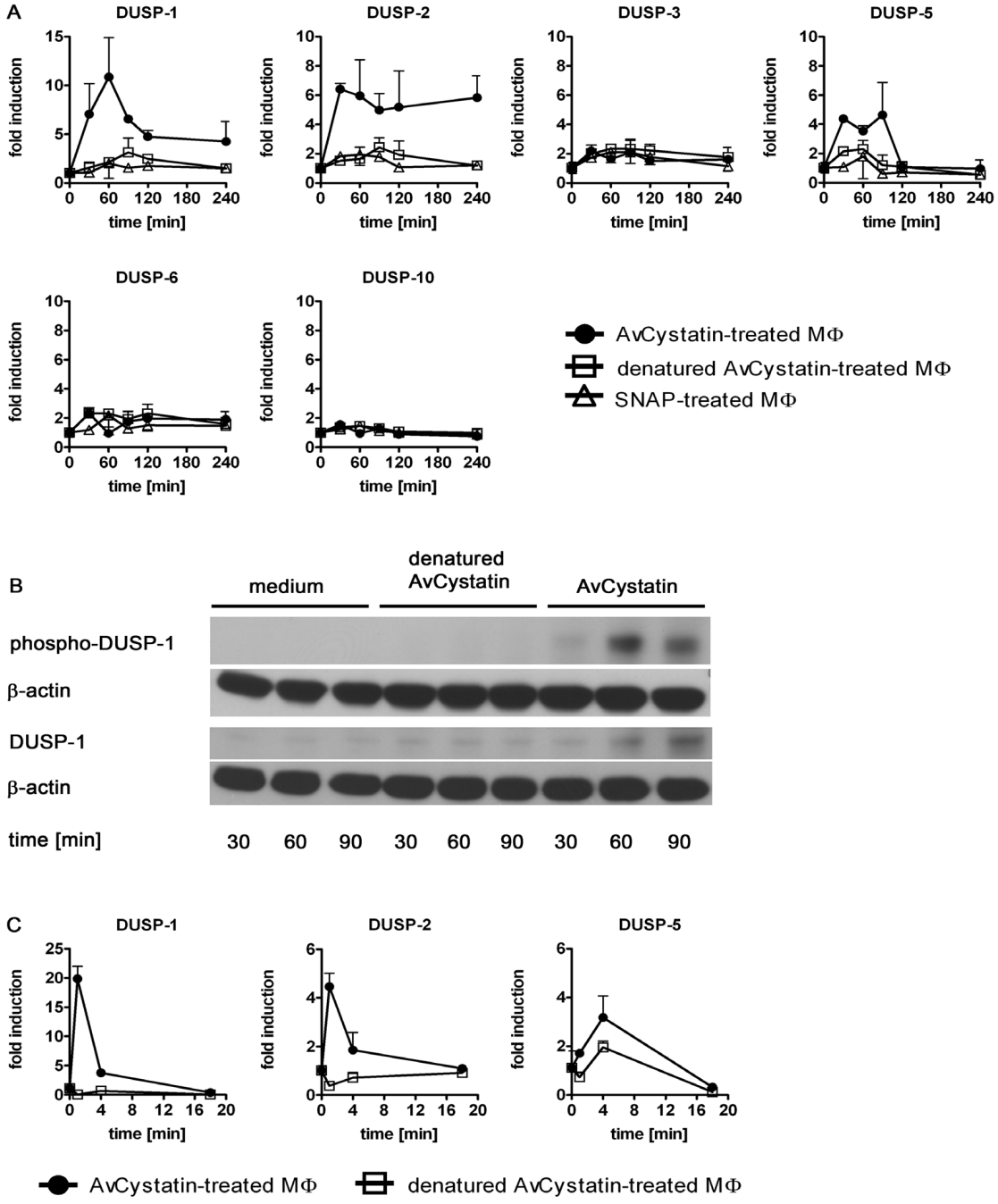

Figure 4-4. AvCystatin induces expression of DUSPs. *In vitro* and *in vivo* expression of dual-specificity phosphatases (DUSPs) in AvCystatin- or control-treated macrophages. (A) Peritoneal macrophages were analysed for expression of DUSP-1, DUSP-2, DUSP-3, DUSP-5, DUSP-6 and DUSP-10 after *in vitro* incubation with AvCystatin or control treatments by real-time PCR. (B) Western blot kinetics of phospho-DUSP-1 and DUSP-1 from total cell extracts after *in vitro* stimulation with AvCystatin or denatured AvCystatin. (C) Real-time PCR for DUSP expression in macrophages of AvCystatin- or control-treated animals. Results (mean ± SEM) represent one of 2-3 experiments. Normalized data is expressed as fold induction compared to untreated control mice. Cells were pooled from 5-6 animals and gene expression analysed in triplicate values.

4.2.4 IL-10 expression in AvCystatin-MΦ is regulated by DUSP-1

To elucidate the inhibitory role of DUSPs on MAPK activation and IL-10 expression in peritoneal macrophages, the experimental findings on AvCystatin-induced DUSP expression were compared to the predictions of a mathematical model published by cooperation partners in Figueiredo et al. (2009). This model was used to perform *in silico* analysis on the relevance of MAPK and DUSPs for IL-10 expression in AvCystatin-MΦ and predicted that an inhibition of p38 would suppress the AvCystatin-induced IL-10 production (Klotz et al., 2011 b). DUSPs exhibit preferences for specific MAPK. Whereas DUSP-1 shows preferential activity for MAPK p38, DUSP-2 appears to hold highest substrate specificity for ERK (Jeffrey et al., 2007; Liu et al., 2007). The experimental findings demonstrated elevated DUSP-1 expression in AvCystatin-MΦ, while the mathematical model predicted IL-10 suppression through inhibition of p38. Thus, MAPK phosphorylation and IL-10 gene expression were analysed in macrophages of DUSP-1-deficient mice and wildtype littermates. Control animals showed transient IL-10 expression after treatment with AvCystatin. In DUSP-1-deficient mice, however, the IL-10 expression was increased and sustained (**Figure 4-5, A**). Western blot analysis determined an accumulation of phospho-p38 and slightly reduced levels of phospho-ERK in DUSP-1-deficient mice (**Figure 4-5, B**). In summary, these findings demonstrate an important role for DUSP-1 in regulating MAPK activation and IL-10 expression in AvCystatin-MΦ.

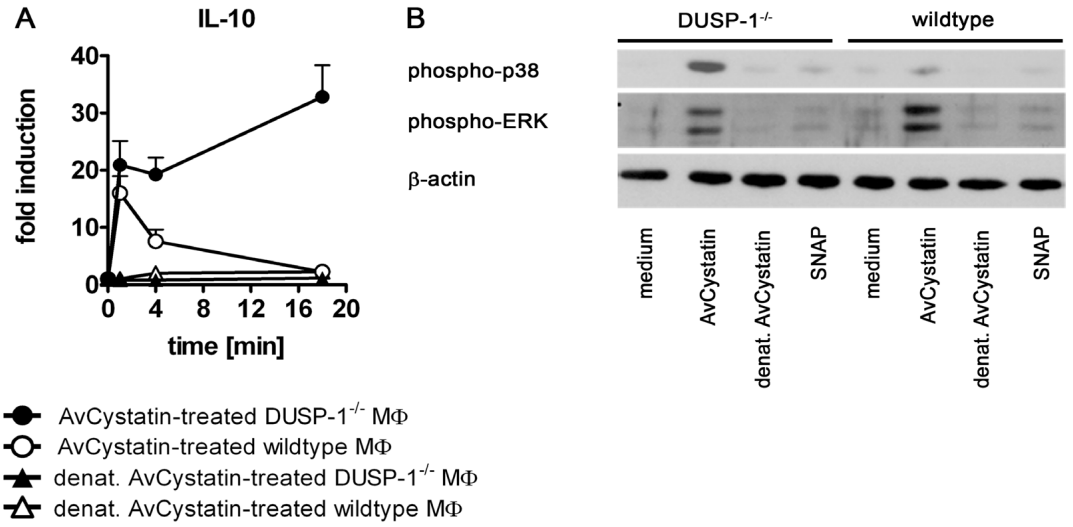

Figure 4-5. DUSP-1 regulates MAPK activation and IL-10 expression in AvCystatin-MΦ. (A) IL-10 expression in macrophages of DUSP-1-deficient mice and wildtype littermates after intraperitoneal injection of 20 µg AvCystatin or denatured AvCystatin. (B) Western blot analysis of phospho-p38 and phospho-ERK in DUSP-1-deficient mice and wildtype littermates. Results are shown as mean ± SEM for one of 2-3 experiments and normalized data is expressed as fold induction compared to untreated control mice. Cells were pooled from 5-6 animals and gene expression analysed in triplicate values.

4.3 Part II: Functional and molecular characteristics of AvCystatin-MΦ

In part II, the functional relevance of AvCystatin-MΦ was examined by addressing whether adoptive transfer of AvCystatin-primed cells interferes with inflammation in two murine disease models and by further evaluating the mechanism of immunosuppression.

4.3.1 Effects of AvCystatin-MΦ in allergic airway hyperreactivity

The induction of airway inflammation with the model allergen ovalbumin is a commonly used method to reflect symptoms of asthma in mice. In contrast to C57BL/6 mice, the BALB/c mouse strain has a genetically determined tendency to develop Th2 responses and is most commonly used to investigate different facets of allergic inflammation (Schröder and Maurer, 2007). By using the model of ovalbumin-induced airway hyperreactivity on a female BALB/c mouse background the present investigation aimed to address whether (a) protective effects of AvCystatin can be transferred by modulated cells through adoptive transfer experiments and (b) whether such effects are dependent on the IL-10 production of transferred cells.

4.3.1.1 Adoptive transfer of AvCystatin-stimulated peritoneal exudate cells

To address whether AvCystatin-primed cells exert beneficial effects on allergic airway inflammation *in vivo*, total PEC were transferred from mice that have been intraperitoneally treated with AvCystatin or control applications. Airway inflammation was induced in female BALB/c mice by using a sensitization and provocation scheme including two intraperitoneal sensitizations with ovalbumin in Imject Alum adjuvant and two intranasal allergen challenges on two consecutive days. PEC were isolated from donor animals 18-20 hours after intraperitoneal administration of 20 µg recombinant AvCystatin or control applications and transferred 4 days prior to intranasal challenge (**Figure 4-6**). Transfer of control-treated cells (stimulated with denatured AvCystatin or SNAP-tag) was performed to exclude possible side effects by the cell transfer alone. Preliminary studies confirmed that transfer of control-treated PEC in ovalbumin-sensitized mice established similar immune responses after antigen-challenge as ovalbumin-sensitized mice without cell transfer (**Figure 4-7**). Compared to control treatments, single intravenous injection of $3x10^6$ AvCystatin-primed PEC (AvCystatin-PEC) significantly reduced total and ovalbumin-specific IgE. However, ovalbumin-specific IgG1 and IgG2a responses were not altered by the cell transfer (**Figure 4-8, A)**. Levels of IL-4, IL-5 and IL-13 in both the BAL-fluid and in culture supernatants of antigen-restimulated splenocytes were drastically reduced in animals treated with AvCystatin-PEC. In contrast, these animals responded with significantly increased levels of local and systemic IL-10 (**Figure 4-8**, **B** and **C**). Analysis of the cell composition in the BAL-

fluid revealed a strong reduction of eosinophil numbers by approximately 40-50% in animals which were treated with AvCystatin-PEC (**Figure 4-8, D**). Moreover, histological analysis of lung sections through periodic acid-Schiff (PAS) and hematoxylin/eosin (HE) staining showed that transfer of AvCystatin-PEC reduced mucus production in the bronchioles and lowered airway cell infiltration (**Figure 4-8, E**). To elucidate whether IL-10 production by cells within the PEC population was responsible for the protective effects, AvCystatin-PEC of IL-10-deficient mice were transferred in parallel. Strikingly, single tail-vein injection of 3x106 AvCystatin-PEC from IL- 10-deficient mice into recipient mice resulted in similar suppression of all analysed parameters compared to the transfer of wildtype AvCystatin-PEC (**Figure 4-8, A-E**). Collectively, these results demonstrate a strong suppressive effect of AvCystatin-PEC on ovalbumin-induced airway hyperreactivity independent of IL-10 expression by the transferred cells.

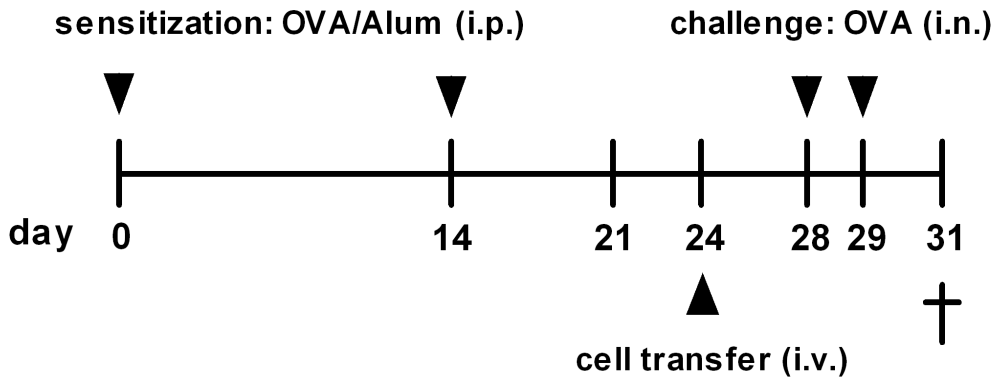

Figure 4-6. Scheme of ovalbumin-induced airway hyperreactivity and cell transfer. BALB/c mice were sensitized with two intraperitoneal (i.p.) injections of 20 µg ovalbumin in Imject Alum adjuvant on day 0 and 14 followed by two intranasal (i.n.) allergen challenges with 50 µg/animal ovalbumin on day 28 and 29. Cells were isolated from mice treated with AvCystatin or denatured AvCystatin 18-20 hours after intraperitoneal injection and adoptively transferred through tail-vein administration into ovalbumin-sensitized mice 4 days (day 24) prior to intranasal provocation with the allergen.

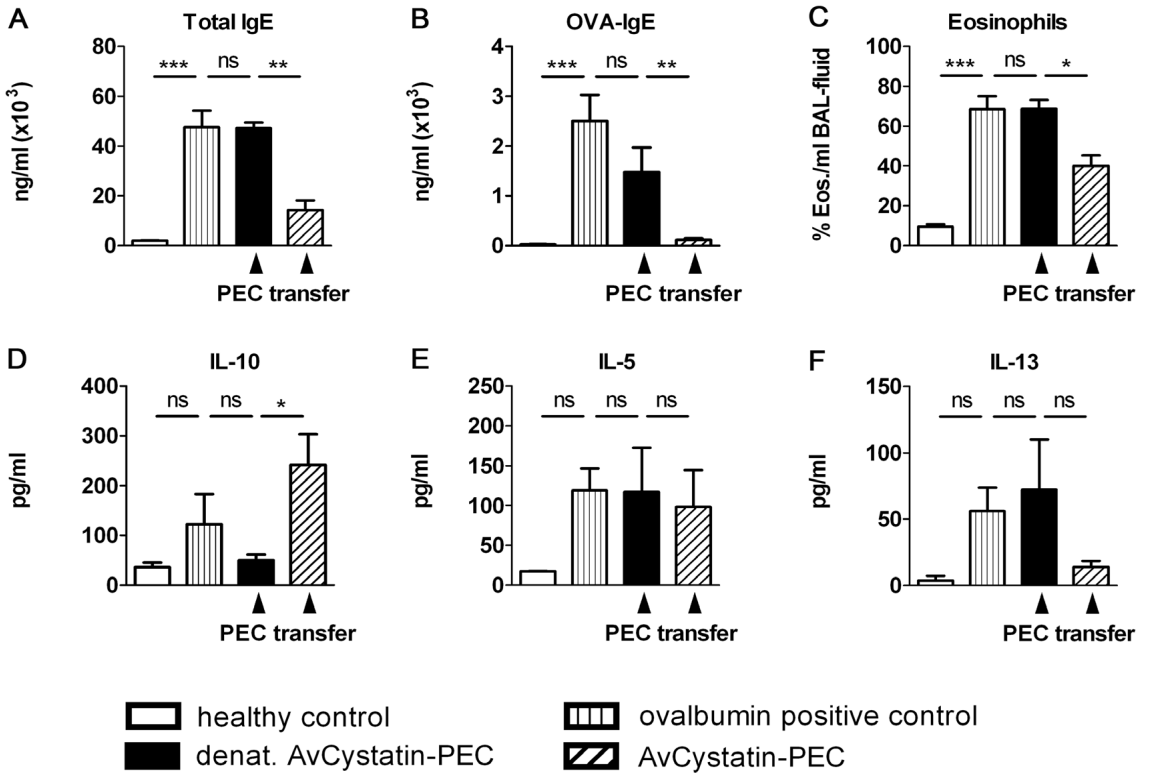

Figure 4-7. Establishment of positive controls for transfer experiments. Female BALB/c mice were sensitized with PBS/Imject Alum and challenged with PBS (healthy control) or sensitized with ovalbumin/Imject Alum and challenged with ovalbumin. For the latter treatment, animals either received no cell transfer (ovalbumin positive control) or were intravenously treated with AvCystatin- or denatured AvCystatin-stimulated peritoneal exudate cells. (A) Total serum IgE. (B) Ovalbumin-specific serum IgE. (C) Percentage of eosinophils in the BAL-fluid. (D-F) Local cytokine response in the BAL-fluid. The figure shows one initial experiment which was performed to establish positive controls for cell transfer experiments. Since no significant differences were detected between the ovalbumin positive control without cell transfer and transfer of control-treated (denat. AvCystatin-PEC) cells, only one control group was used for following transfer experiments.

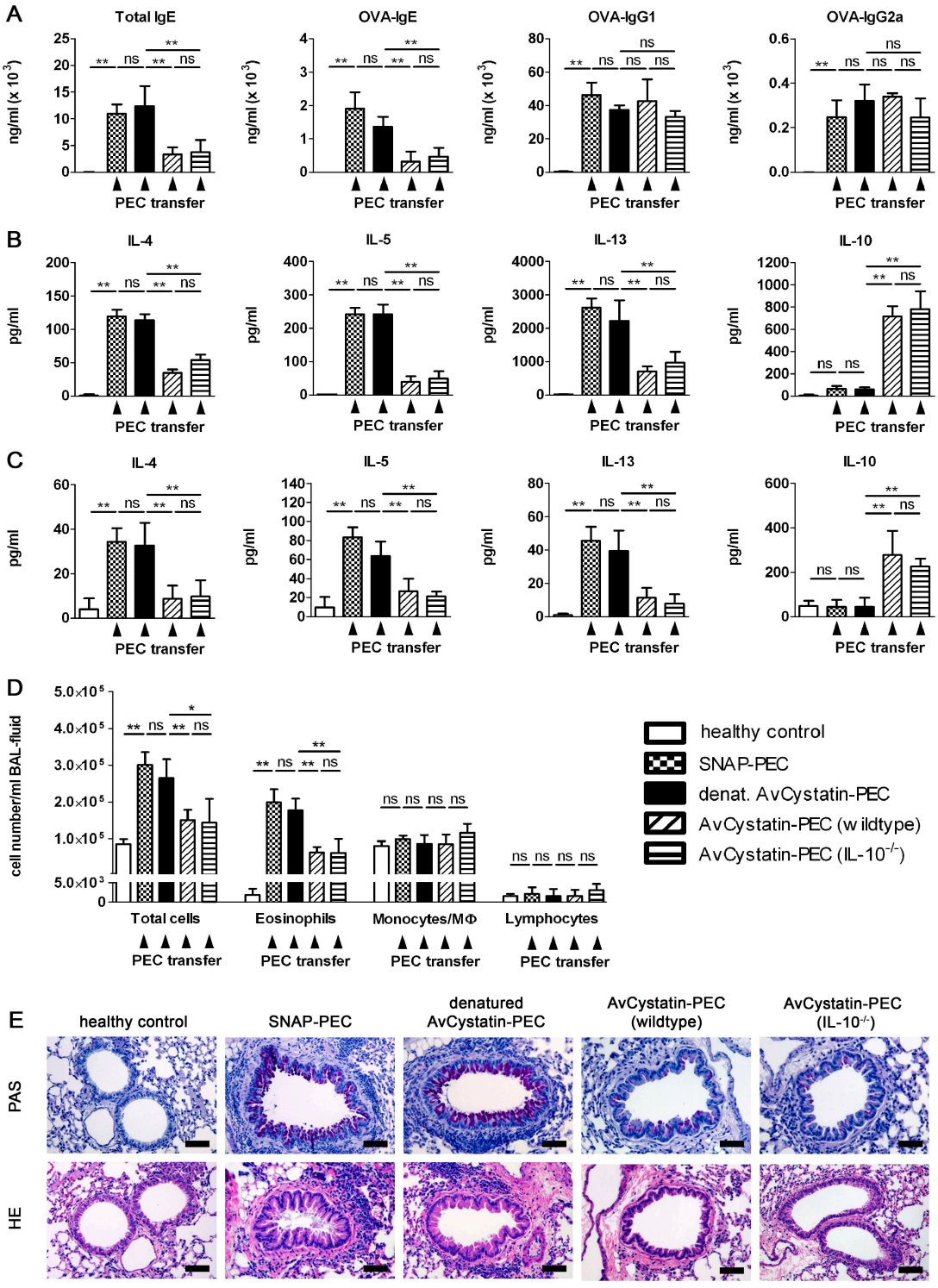

Figure 4-8. AvCystatin-primed PEC protect against airway inflammation. Peritoneal exudate cells were isolated from BALB/c wildtype or IL-10-deficient mice 18-20 hours after intraperitoneal injection of AvCystatin or denatured AvCystatin. $3x10^6$ cells were intravenously transferred into ovalbumin-sensitized mice prior to intranasal challenge with ovalbumin. (A) Total and allergen-specific levels of serum IgE and ovalbumin-specific IgG1 and IgG2a in recipient mice. (B) Systemic cytokine response in culture supernatants of allergen-restimulated recipient splenocytes. (C) Local cytokine response in the BAL-fluid of recipients measured by quantification of IL-4, IL-5, IL-13 and IL-10. (D) Cell counts for inflammatory cells in the BAL-fluid. (E) Histological staining of lung bronchioles with PAS and HE. Bars, 100 µm. Results are presented as mean ± SEM for two independent experiments with 5-6 animals per group. *, $P < 0.05$; **, $P < 0.01$.

4.3.1.2 Transfer of AvCystatin-MΦ abrogates allergic airway inflammation

To determine the suppressive potential of AvCystatin-MΦ on airway inflammation, macrophages were purified from the PEC population of donor mice 18-20 hours after treatment with AvCystatin or denatured AvCystatin. Purity of untouched macrophages was > 95% as revealed by flow cytometry analysis for the expression of macrophage markers F4/80 and CD11b (**Figure 4-9, A**). Single intravenous transfer of $2x10^6$ macrophages 4 days prior to intranasal provocation (**Figure 4-6**) reduced the total and ovalbumin-specific IgE response of recipient mice but did not affect antigen-specific serum IgG1 and IgG2a (**Figure 4-9, B**). Local and systemic levels of IL-4, IL-5 and IL-13 were significantly reduced by treatment with AvCystatin-MΦ. On the contrary, elevated levels of IL-10 in the BAL-fluid and in splenocyte culture supernatants were detected (**Figure 4-9, C** and **D**). Eosinophil infiltration was significantly hampered through the treatment with AvCystatin-MΦ (**Figure 4-9, E**) and associated with reduced expression of eotaxin-1, eotaxin-2, CCR3 and MCP-5 in lung homogenates (**Figure 4-9, F**). The expression of other chemokines such as MCP-1 (CCL2), VCAM-1 (CD106) or RANTES (CCL5) was not altered by the transfer of AvCystatin-MΦ (data not shown). Histological analysis of lung sections by PAS and HE staining revealed mitigated mucus production and inflammatory cell infiltration after treatment with AvCystatin-MΦ (**Figure 4-9, G**). To assess whether AvCystatin-MΦ survive in recipient animals, $2\text{-}3x10^6$ carboxyfluorescein succinimidyl ester (CSFE)-labelled macrophages were intravenously transferred on day 24 into ovalbumin-sensitized animals prior to challenge and their distribution analysed in the lung, BAL-fluid, peribronchial lymph nodes (PBLNs) and the spleen of recipient animals on day 31 by flow cytometry. Labelled macrophages were detected in the spleen (AvCystatin-MΦ: 194491 ± 27549 cells; control-treated MΦ: 94680 ± 10013 cells; mean ± SEM) and to a lower amount in the PBLNs (AvCystatin-MΦ: 3985 ± 2870; control-treated MΦ: 1824 ± 202; mean ± SEM) of recipient mice (**Figure 4-9, H**). Hence, these findings show that AvCystatin-MΦ efficiently protect against allergic airway inflammation and reside for at least seven days in treated animals.

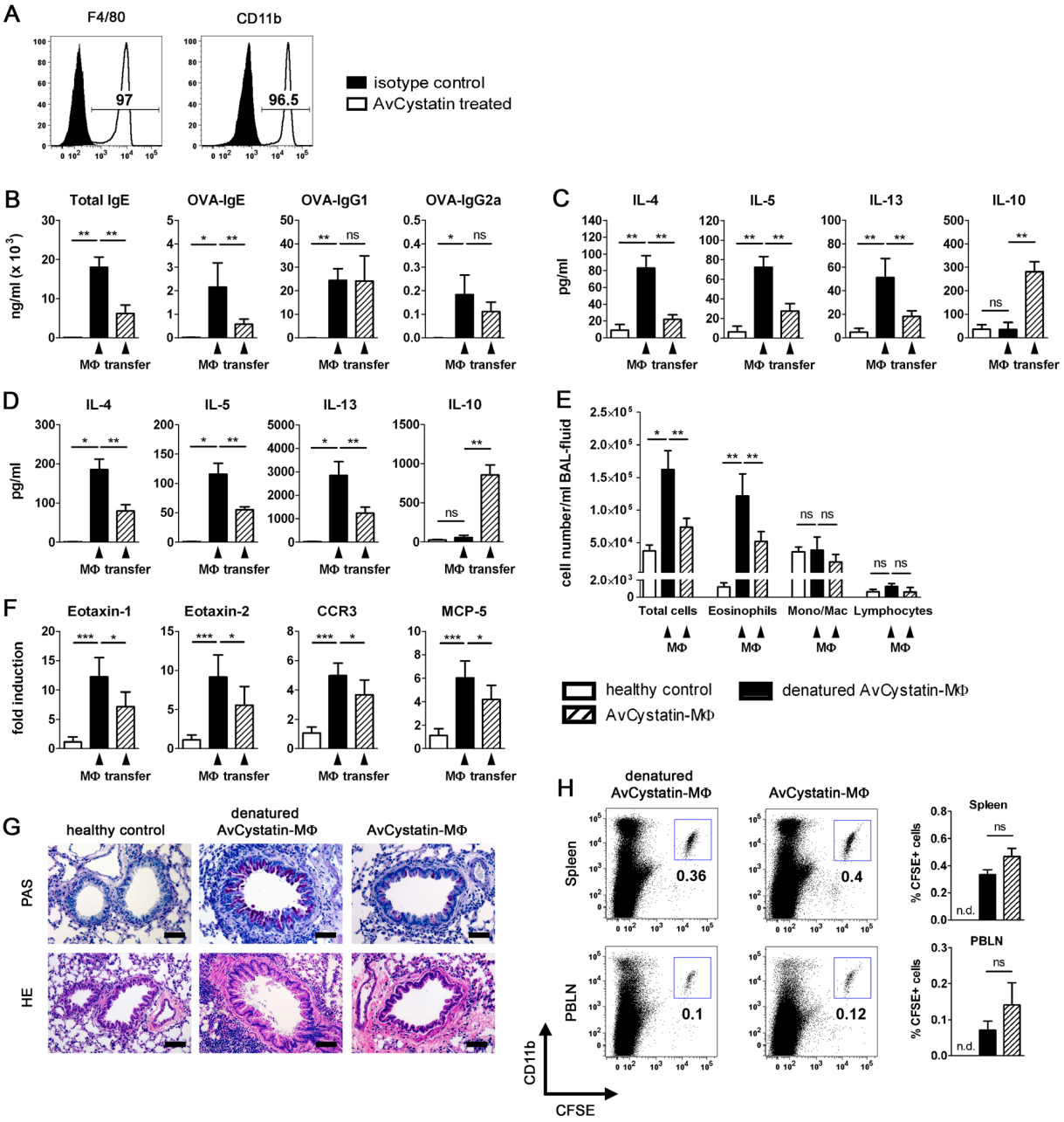

Figure 4-9. AvCystatin-MΦ ameliorate allergic airway inflammation. Macrophages were purified from peritoneal exudate cells of AvCystatin- or control-treated BALB/c mice 18-20 hours after intraperitoneal injection and intravenously transferred ($2x10^6$) in ovalbumin-sensitized mice prior to airway challenge. (A) Purity of enriched AvCystatin-MΦ was determined by expression of F4/80 and CD11b (both > 95%). Similar values were detected for control-treated macrophages (data not shown). (B) Levels of total and allergen-specific IgE, ovalbumin-IgG1 and -IgG2a in recipient mice. (C) Concentrations of IL-4, IL-5, IL-13 and IL-10 in the BAL-fluid. (D) Antigen-specific cytokine response in splenocyte culture supernatants. (E) Analysis of total cell numbers, eosinophils, monocytes/macrophages and lymphocytes in the BAL-fluid. (F) Analysis of chemokine expression in lung homogenates by quantitative real-time PCR. Gene expression was analysed in triplicate values and expressed as fold induction compared to untreated control mice. (G) Histological analysis of mucus production and inflammatory cell influx in bronchioles by PAS and HE staining. Bars, 100 µm. (H) Detection of CFSE-labelled macrophages in the spleen and in the PBLNs of recipient mice by flow cytometry analysis on day 31. Values are presented as mean ± SEM of two independent experiments with 5-6 mice per group for (B-G) and 3 mice per group for (H). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4.3.1.3 B cell transfer does not suppress airway inflammation

Recent publications show helminth-induced regulatory B cells to be capable of abrogating symptoms of airway inflammation by adoptive cell transfer (Mangan et al., 2004; Amu et al., 2010; Wilson et al., 2010). After intraperitoneal application of AvCystatin, B cells accounted for 30-40% of the PEC population. In order to evaluate whether AvCystatin-stimulated B cells within the peritoneal cell population mediated immunosuppression, $2x10^6$ purified B cells (purity 90-95%) of AvCystatin- or control-treated mice were intravenously administered into ovalbumin-sensitized animals by using the same transfer protocol as described previously (**Figure 4-6**). Compared to intravenous application of AvCystatin-PEC or AvCystatin-MΦ, the administration of B cells did not transfer protective effects on any of the investigated parameters such as IgE levels (**Figure 4-10, A**), local and systemic cytokine responses (**Figure 4-10, B** and **C**), the cell composition in the BAL-fluid (**Figure 4-10, D**) or mucus production (**Figure 4-10, E**).

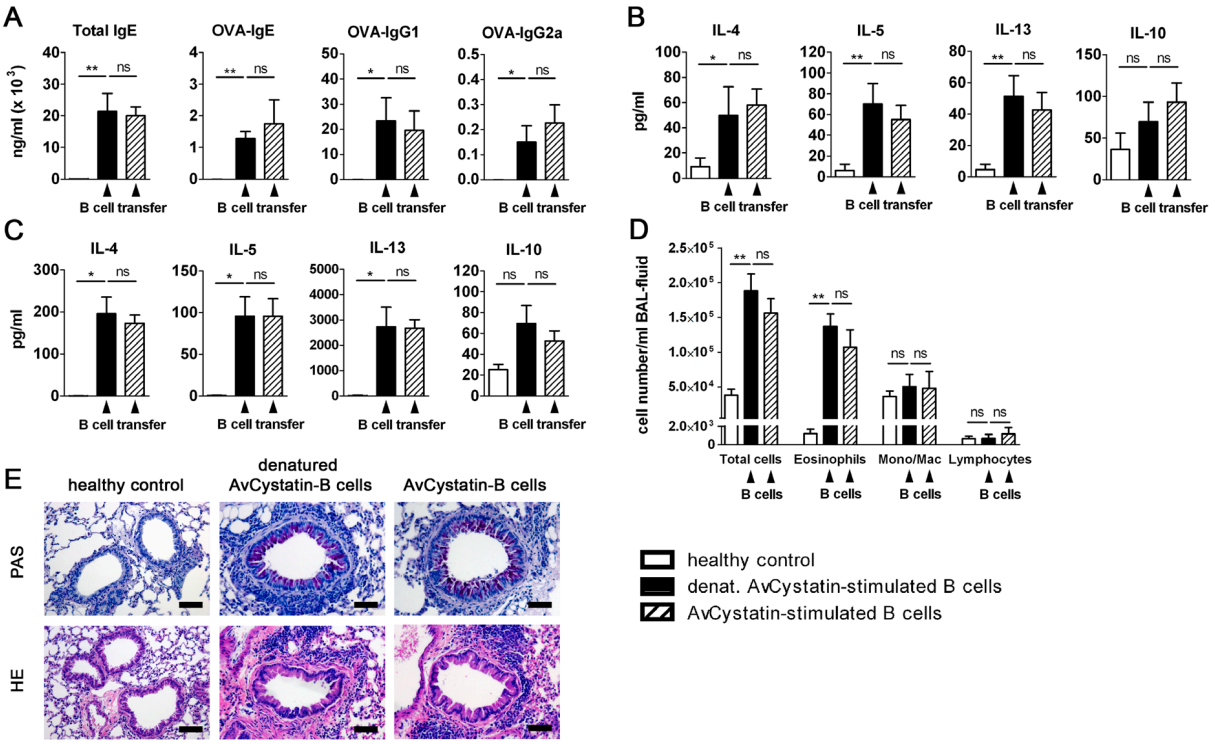

Figure 4-10. Transfer of B cells does not protect against airway inflammation. $2x10^6$ purified B cells of AvCystatin- or control-treated mice were intravenously transferred into ovalbumin-sensitized mice 18-20 hours after injection of the immunomodulator. (A) Total and allergen-specific IgE, ovalbumin-IgG1 and -IgG2a. (B) Th2 cytokine and IL-10 response in BAL-fluid. (C) Antigen-specific cytokine response of recipient splenocytes. (D) Analysis of inflammatory cells in the BAL-fluid. (E) PAS and HE staining of lung sections. Bars, 100 µm. Results are presented as mean ± SEM for one representative experiment with 5-6 mice per group. *, $P < 0.05$; **, $P < 0.01$

4.3.1.4 AvCystatin-MΦ *in vivo* and *in vitro* induce IL-10-producing T cells

The induction of IL-10-secreting immunoregulatory cells is a common feature of helminth immunomodulation. Previous studies determined AvCystatin to reduce airway hyperreactivity in a macrophage-mediated and IL-10-dependent manner (Schnoeller et al., 2008). In the present study, AvCystatin-MΦ significantly increased local and systemic IL-10 in recipient mice. However, elevated IL-10 levels were detected after transfer of AvCystatin-primed cells from IL-10-deficient mice as well. Thus, an indirect suppressive effect of AvCystatin-MΦ through the induction of an IL-10-producing cell type was hypothesized. $CD4^+$ T cells have been described as important producers of IL-10 in diverse contexts of helminth infections or after treatment with helminth-secreted products. Therefore, $CD4^+$ T cells were purified by negative depletion (purity ≥ 95%) from the spleen of healthy control mice or ovalbumin-sensitized and -challenged animals that had been treated with AvCystatin- or control-stimulated macrophages prior to intranasal provocation. ConA stimulation of purified $CD4^+$ T cells significantly up-regulated the expression of IL-10 transcript in $CD4^+$ T cells (**Figure 4-11, A** upper panel) and increased levels of IL-10 in $CD4^+$ T cell culture supernatants (**Figure 4-11, A** lower panel). Analysis of Foxp3 expression in IL-10-producing $CD4^+$ T cells showed no differences between experimental groups (data not shown). To verify the induction of IL-10-producing $CD4^+$ T cells by AvCystatin-MΦ, an *in vitro* system was established reflecting the *in vivo* situation by incubating macrophages with ovalbumin-specific $CD4^+$ T cells of DO11.10 mice and bone marrow-derived dendritic cells pre-treated with the ovalbumin-peptide 323-339 (OVA-DC). To investigate whether IL-10 induction in $CD4^+$ T cells is cell contact-dependent or mediated through soluble factors, *in vitro* assays were performed in a mixed population and transwell approach separating the macrophages from T cells and dendritic cells. In both cases AvCystatin- but not control-treated macrophages clearly induced IL-10 in $CD4^+$ T cells (**Figure 4-11, B** upper and middle panel). Moreover, $CD4^+$ T cell and OVA-DC co-cultures in the presence of AvCystatin- but not control-treated macrophage-supernatants (MΦ-S) showed significantly increased IL-10 levels in the culture supernatants (**Figure 4-11, B** lower panel) suggesting a cell contact-independent mechanism for the observed IL-10 induction. IL-10 was not detected in co-cultures of $CD4^+$ T cells, macrophages and untreated dendritic cells (**Figure 4-11, B** upper and middle panel). Moreover, incubation of $CD4^+$ T cells with ovalbumin-peptide-treated AvCystatin-MΦ in the absence of dendritic cells did not induce IL-10 production in $CD4^+$ T cells (data not shown). Intracellular staining of IL-10 finally verified $CD4^+$ T cells as the cellular origin for IL-10 in both the mixed population and transwell approach (**Figure 4-11, C**).

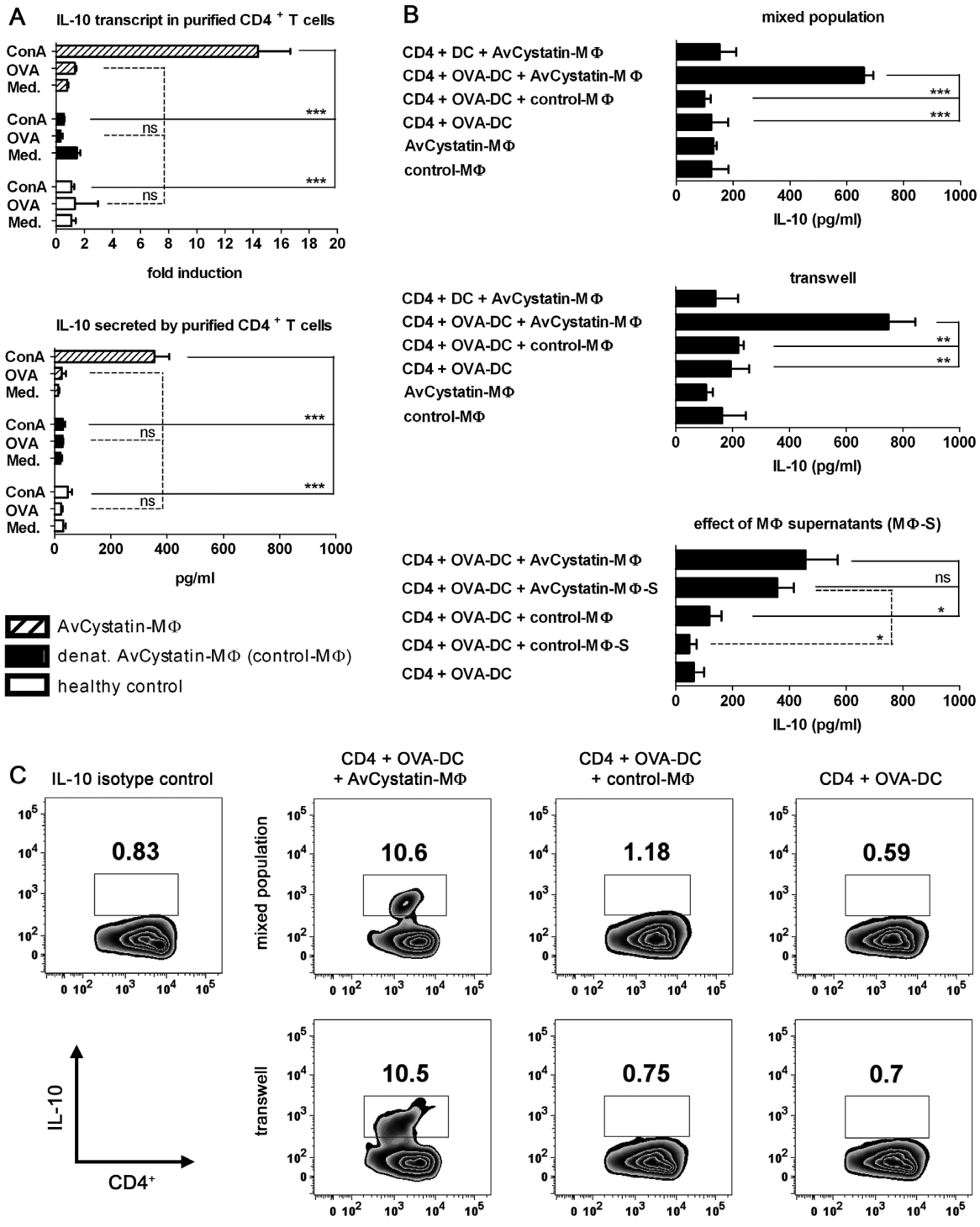

Figure 4-11. AvCystatin-MΦ induce CD4$^+$IL-10$^+$ T cells. (A) Splenic CD4$^+$ T cells were purified from healthy mice or allergic animals after treatment with AvCystatin-MΦ or denatured AvCystatin-treated macrophages (control-MΦ). IL-10 transcript (upper panel) and IL-10 protein (lower panel) was analysed after stimulation with ovalbumin or ConA. Results (mean ± SEM) are shown for one of two independent experiments with 5-6 mice per group. Normalized data is expressed as fold induction compared to healthy control mice. ***, $P < 0.001$. (B) Detection of IL-10 in culture supernatants after incubation of *in vivo*-generated AvCystatin-MΦ or control-MΦ with CD4$^+$ T cells and dendritic cells. Assays were performed in a mixed population and transwell approach or CD4$^+$ T cells and dendritic cells were alternatively incubated with macrophage supernatants (MΦ-S). (C) Intracellular staining of IL-10 in CD4$^+$ T cells after *in vitro* co-culture with dendritic cells and macrophages. Data represents mean ± SEM for one of at least three independent experiments. Triplicate analysis was performed for pooled cells of 3-6 mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4.3.1.5 AvCystatin-MΦ impair CD4$^+$ T cell cytokine production

To evaluate the impact of AvCystatin-MΦ on other ovalbumin-specific CD4$^+$ T cell cytokines, levels of IL-2, IFN-γ and IL-13 were assessed in co-culture supernatants of macrophages, dendritic cells and CD4$^+$ T cells. Incubation of dendritic cells and CD4$^+$ T cells in the absence of macrophages induced elevated levels of IL-2 (**Figure 4-12, B**), IFN-γ (**Figure 4-12, C**) and IL-13 (**Figure 4-12, D**). IL-10, however, was not detectable (**Figure 4-12, A**). Compared to control-treated macrophages which did not affect cytokine induction through dendritic cell-mediated T cell activation, AvCystatin-MΦ significantly suppressed levels of IL-2, IFN-γ and IL-13 (**Figure 4-12, B-D**) but increased the amount of IL-10 (**Figure 4-12, A**). To examine whether the CD4$^+$ T cell-derived IL-10 affects T cell cytokine responses through an autocrine feedback, ovalbumin-specific CD4$^+$ T cells were pre-treated with 10 µg/ml anti-IL-10 receptor antibody (clone 1B1; DRFZ, Berlin, Germany) for one hour to ensure that IL-10 cannot bind to and signal through its receptor on T cells. Thereafter, CD4$^+$ T cells were co-cultured with ovalbumin-peptide-treated dendritic cells and AvCystatin- or control-treated macrophages in a mixed population approach. Pre-treatment of CD4$^+$ T cells with the anti-IL-10 receptor antibody had no effect on the suppression of IFN-γ and IL-13 (**Figure 4-12, C** and **D**). IL-2 secretion, however, was significantly restored but did not reach the initial level determined in supernatants of CD4$^+$ T cell and dendritic cell co-cultures in the absence of macrophages (**Figure 4-12, B**). Together, these findings show that AvCystatin-MΦ modulate ovalbumin-specific CD4$^+$ T cell responses but exclude an IL-10 autocrine feedback mechanism for the observed suppression of IFN-γ and IL-13 in CD4$^+$ T cells. However, IL-10 seems, at least in part, to be responsible for suppressive effects on IL-2.

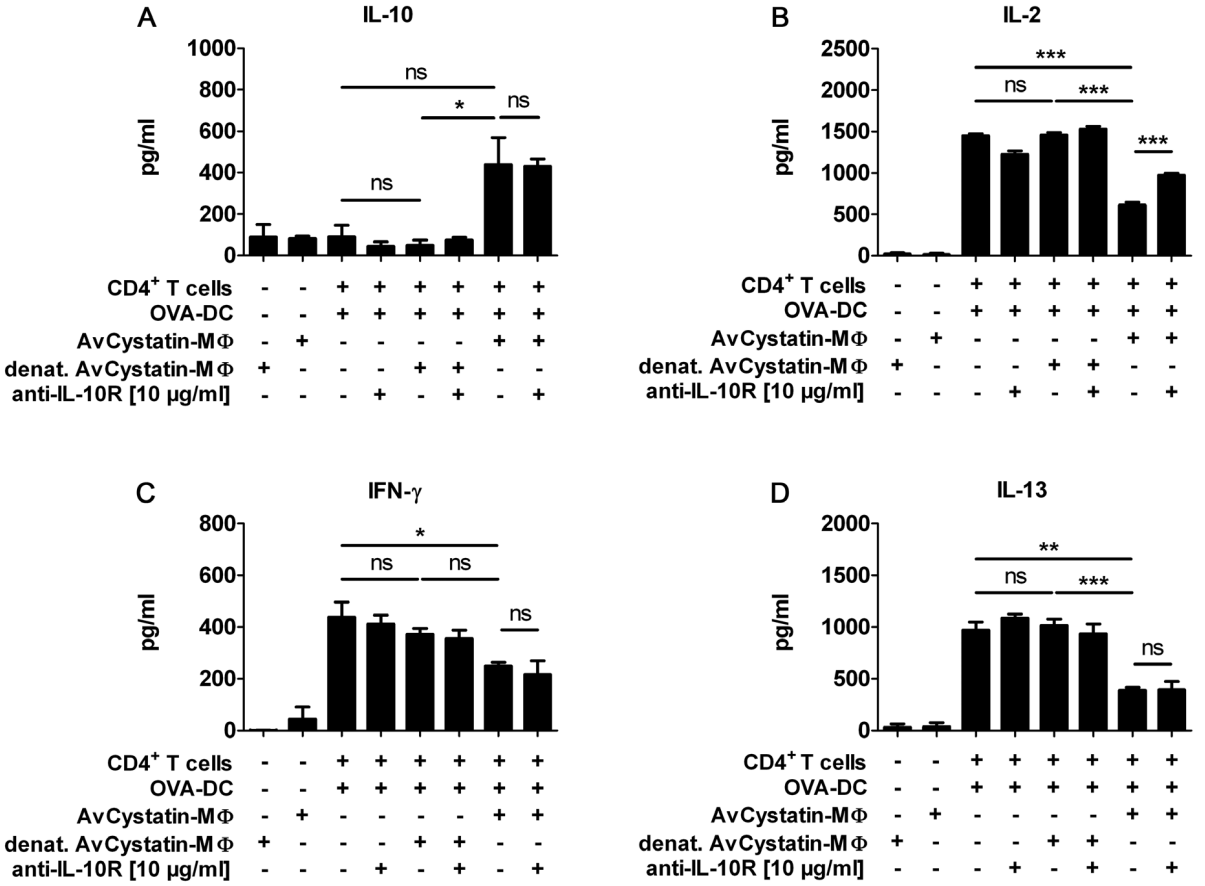

Figure 4-12. Effect of AvCystatin-MΦ on CD4$^+$ T cell cytokine production *in vitro*. *In vivo*-generated macrophages were purified from BALB/c mice and *in vitro* co-cultured with ovalbumin-peptide-treated dendritic cells (OVA-DC) and ovalbumin-specific CD4$^+$ T cells. In parallel, CD4$^+$ T cells were pre-treated with 10 µg/ml anti-IL-10 receptor antibody for one hour and thereafter incubated with dendritic cells and macrophages. Supernatants were collected and analysed by ELISA for IL-10 (A), IL-2 (B), IFN-γ (C) and IL-13 (D). Data is presented as mean ± SEM for one of two independent experiments. Triplicate analysis was performed with pooled macrophages of 5 animals per treatment, purified splenic CD4$^+$ T cells of 3 DO11.10 mice and bone marrow-derived dendritic cells of one animal. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4.3.2 Effects of AvCystatin-MΦ in a model of DSS-induced colitis

To address whether AvCystatin-MΦ transfer protection in another disease setting, macrophages were evaluated in a murine model of dextran sodium sulfate (DSS)-induced colitis. DSS directly exerts toxic effects on colonic epithelial cells of the basal crypts and is commonly used to study innate immune mechanisms in colitis (Wirtz et al., 2007). Disruption of the mucosal barrier by DSS leads to severe histological damage associated with high levels of pro-inflammatory cyokines and the infiltration of granulocytes such as neutrophils and eosinophlis. In the present study, animals were treated with 3% DSS in drinking water for a total of 8 days and $3x10^6$ AvCystatin- or control-treated macrophages were intravenously transferred one day after access to DSS (**Figure 4-13, A**). Single transfer of AvCystatin-MΦ protected recipient mice against body weight loss (**Figure 4-13, B**) and colon shortening, a hallmark symptom of colitis wich arises from longitudinal muscle spasm or irreversible fibrosis (**Figure 4-13, C**). Histological analysis of distal colon sections revealed significantly lowered scores for tissue damage and inflammation after transfer of AvCystatin-MΦ (**Figure 4-13, D** and **E**). Compared to control-treated macrophages, injection of AvCystatin-MΦ suppressed the influx of neutrophils ($MPO7^+$) and eosinophils ($SiglecF^+$) to the submucosa of distal colon (**Figure 4-13, F** and **G**) but did not induce $Foxp3^+$ regulatory T cells or IL-10 (data not shown). Hence, transfer of AvCystatin-MΦ not only protects against airway inflammation but also suppresses DSS-induced colitis.

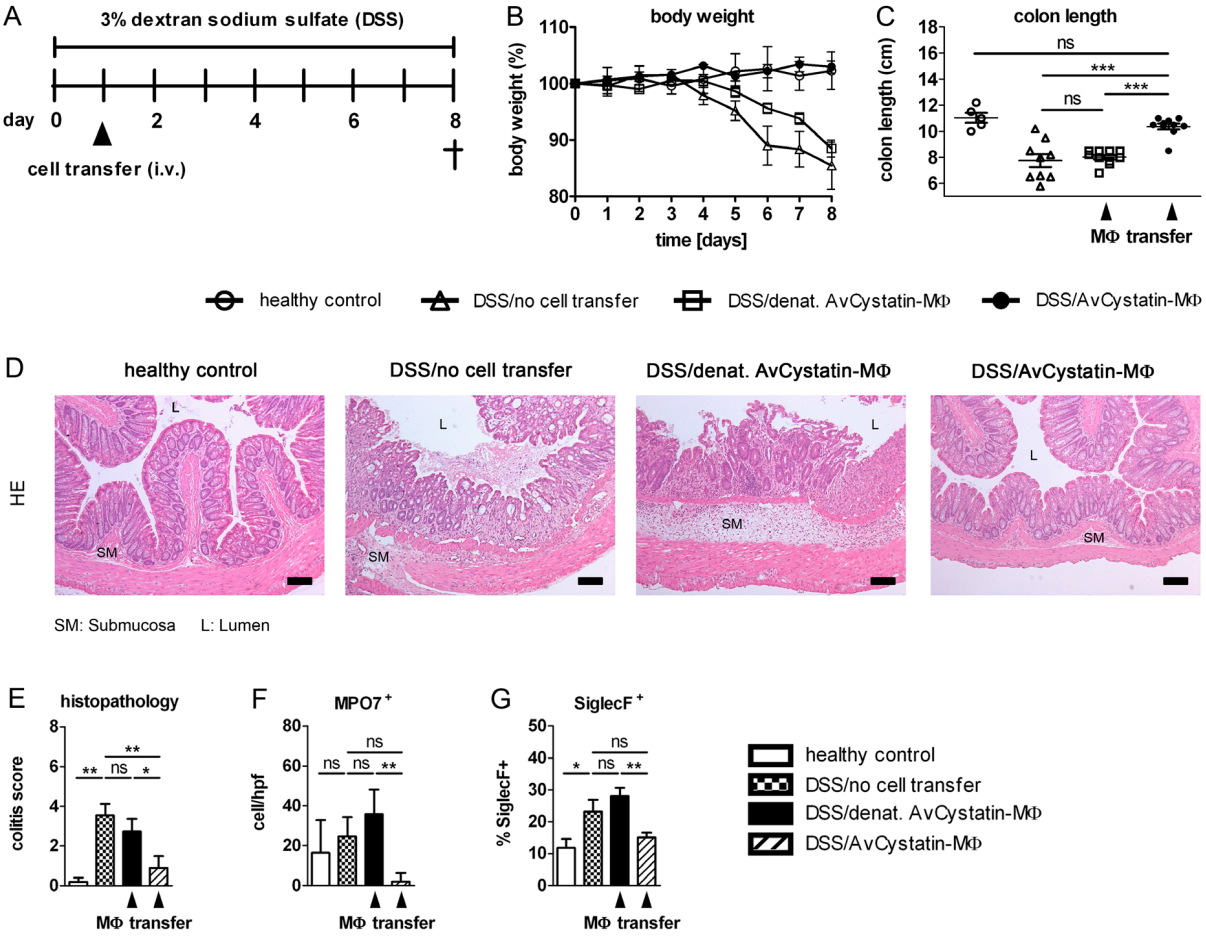

Figure 4-13. AvCystatin-MΦ protect against DSS-induced colitis. $3x10^6$ AvCystatin- or control-treated macrophages of C57BL/6 donor mice were transferred into C57BL/6 recipient animals one day after access to dextran sodium sulfate (DSS) and animals sacrificed on day 8. (A) Scheme of DSS-induced colitis. (B) Body weight curve. (C) Analysis of colon length on day 8. (D) Histological images of HE-stained colon sections. Bars, 100 µM. (E) Histopathology (includes analysis of inflammatory cell influx and scoring of tissue damage). (F) Cell counts of neutrophils ($MPO7^+$) in the submucosal compartment. (G) Flow cytometry analysis of eosinophils ($SiglecF^+$) in the submucosa of distal colon. Pooled data of two independent experiments with 5-9 mice per group is presented as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4.3.3 Phenotyping of AvCystatin-MΦ

In order to characterize the AvCystatin-induced macrophage population and its suppressive activity in more detail, AvCystatin-MΦ were phenotypically examined for gene and surface marker expression.

4.3.3.1 Analysis of the transcriptional macrophage signature

Transcriptional macrophage profiling was performed by analysing selected differentiation markers for murine M1 (iNOS, IL-12/23p40, TNF-α, IL-6), M2a (TGF-β, ARG-1, Ym1, RELM-α), M2b (IL-10, SPHK-1, LIGHT, CCL1) and M2c (TLR-8, IL-21R) activation by quantitative real-time PCR. To elucidate the *in vitro* effect of AvCystatin on macrophage gene expression, enriched macrophages were stimulated with 0.5 µM recombinant or denatured AvCystatin. Analysis of marker gene expression revealed an early and transient expression of pro- and anti-inflammatory marker genes such as inducible nitric oxide synthase (iNOS), IL-12/23p40, TNF-α, IL-6 and IL-10 by macrophages treated with AvCystatin. These markers were down-regulated to background levels 10-18 hours after treatment. Instead, AvCystatin-MΦ changed their phenotype and expressed elevated levels of SPHK-1, LIGHT and ARG-1 (**Figure 4-14, A**). However, treatment with AvCystatin did not induce expression of M2c marker genes (data not shown). To confirm the AvCystatin effect *in vivo*, 20 µg/animal AvCystatin or denatured AvCystatin was intraperitoneally injected into mice. Real-time PCR analysis of purified macrophages reflected the *in vitro* results by showing an early and transient expression of pro- and anti-inflammatory markers followed by expression of SPHK-1, LIGHT and ARG-1 between 5-18 hours after stimulation (**Figure 4-14, B**). Thus, stimulation of peritoneal macrophages with AvCystatin *in vitro* and *in vivo* induces a specific phenotype characterized by an early and transient expression of pro- and anti-inflammatory activation markers followed by a conversion towards a suppressive M2a/M2b hybrid phenotype.

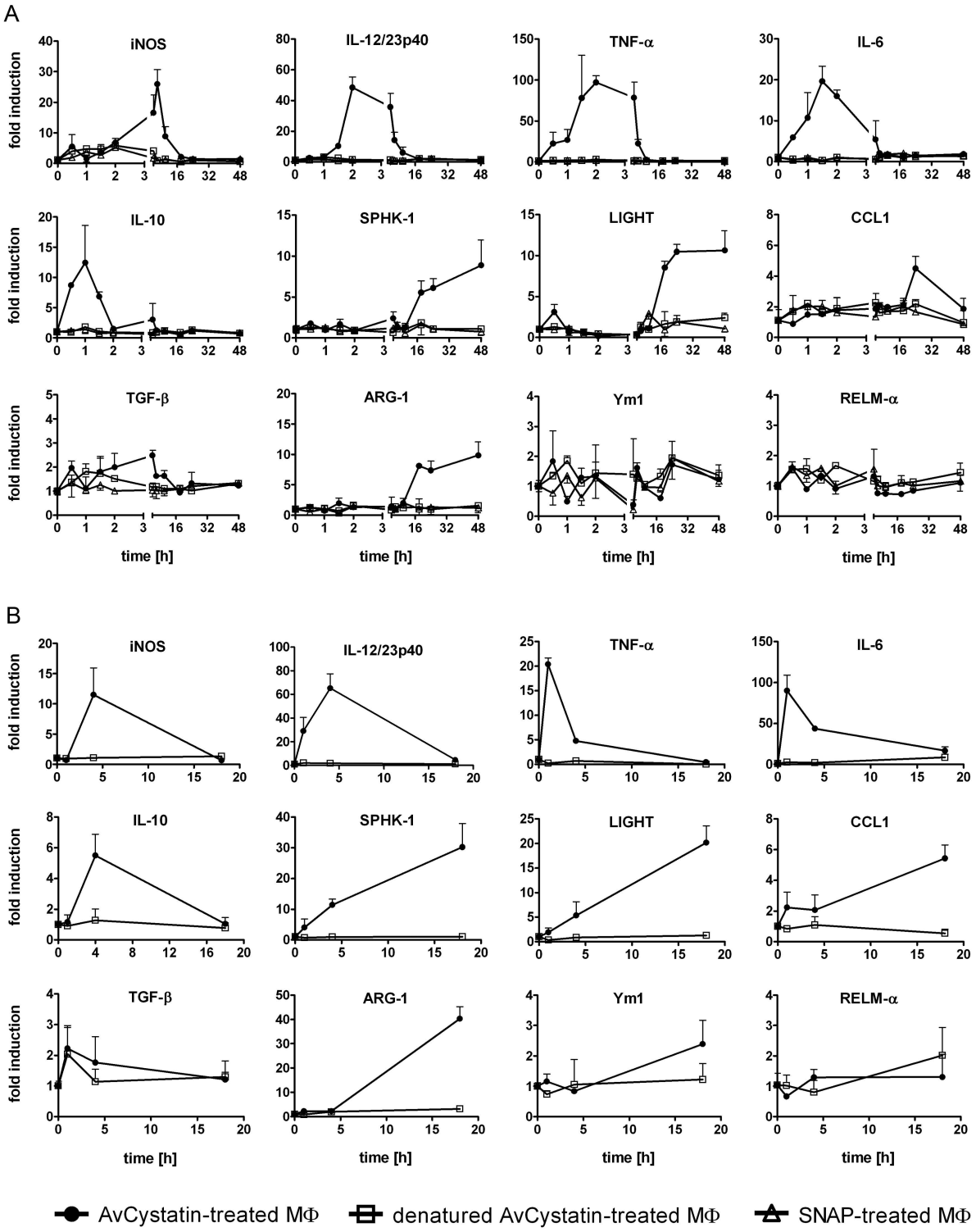

Figure 4-14. Transcriptional characterization of AvCystatin-MΦ. Macrophages were stimulated *in vitro* (0.5 µM) or *in vivo* (20 µg/mouse) with AvCystatin or control treatments. (A) Real-time PCR analyses for the expression of selected macrophage activation markers after *in vitro* stimulation. Results (mean ± SEM) are representative for one of three independent experiments. (B) Analysis of marker expression in macrophages after *in vivo* stimulation. Results (mean ± SEM) are representative for one of two independent experiments. Normalized data is expressed as fold induction compared to untreated control animals. For each group, macrophages of 8-10 animals were pooled and gene expression analysed in triplicate values.

4.3.3.2 Phenotypic conversion of AvCystatin-MΦ is IL-10 independent

It was formerly shown that M2 macrophages can inhibit M1 macrophage activation through the secretion of IL-10 (Katakura et al., 2004). To assess whether an autocrine effect of AvCystatin-MΦ-secreted IL-10 induces a phenotypic shift from M1 to M2a/M2b activation, the expression of selected marker genes was examined in macrophages of wildtype and IL-10-deficient mice at an early and late timepoint after treatment with AvCystatin or denatured protein. No significant differences were observed between macrophages of wildtype and IL-10-deficient mice. *In vivo* stimulation with AvCystatin induced early up-regulaton of iNOS, IL12/23p40, TNF-α and IL-6 in macrophages of wildtype and IL-10-deficient mice. As expected, IL-10 was only induced in wildtype but not in IL-10-deficient macrophages. Early marker genes were down-modulated to background levels 18-20 hours post stimulation. Instead, the expression of SPHK-1, LIGHT and ARG-1 was detectable in both wildtype and IL-10-deficient macrophages (**Figure 4-15**). These results show that the phenotypical conversion of AvCystain-MΦ from M1 activation towards an M2a/M2b signature does not depend on an autocrine IL-10 effect.

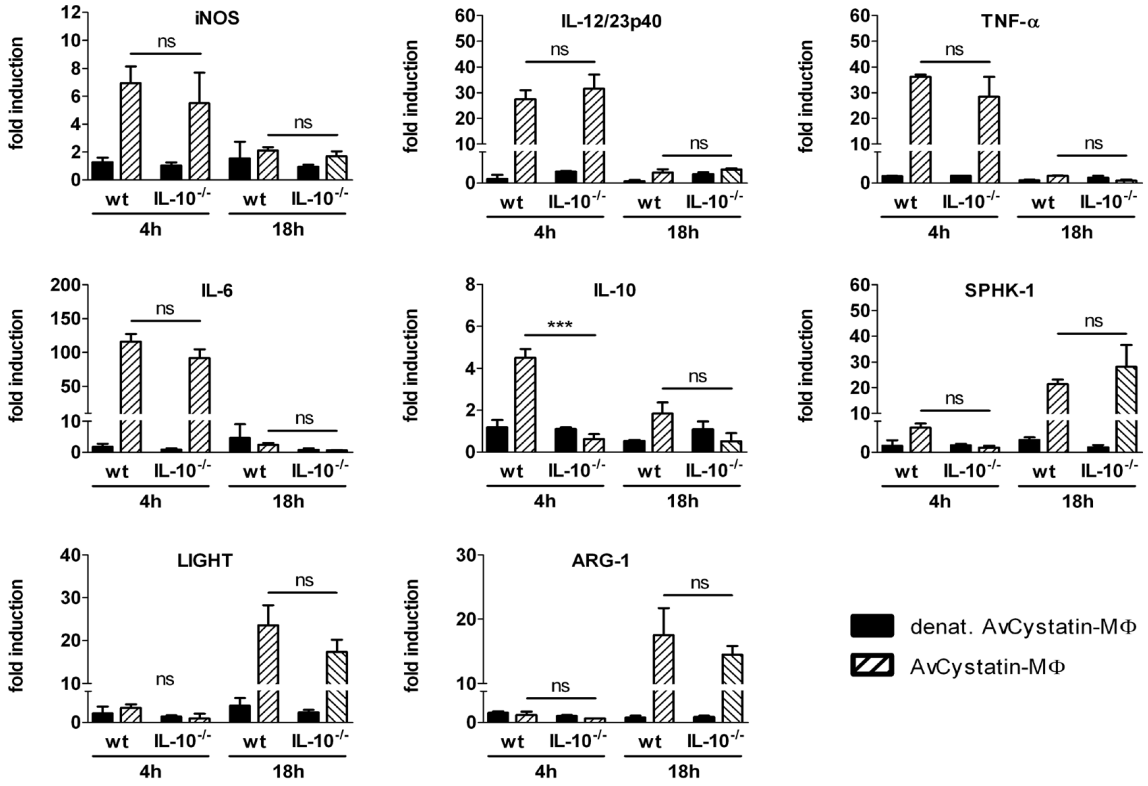

Figure 4-15. IL-10 does not induce phenotypic conversion of AvCystatin-MΦ. Macrophages of wildtype (wt) or IL-10-deficient (IL-10$^{-/-}$) mice were analysed for their marker expression after *in vivo* treatment with AvCystatin or denatured AvCystatin. Results (mean ± SEM) are shown for one experiment. Normalized data is expressed as fold induction compared to untreated control mice. Macrophages of 5-6 animals per group were pooled and gene expression analysed in triplicate values. ***, P < 0.001.

4.3.3.3 AvCystatin-MΦ surface marker profile

To assess the effect of AvCystatin on macrophage surface molecules, the expression of surface markers of *in vivo*-generated AvCystatin-MΦ was determined 18-20 hours after intraperitoneal treatment with the immunomodulator. Flow cytometric analysis of peritoneal F4/80$^+$CD11b$^+$ AvCystatin-MΦ revealed up-regulation of MHC-II, CD40, CD80, CD86, Fcγ receptor (FcγR) III and II (CD16/32), PD-L1 (CD274), PD-L2 (CD273) and intercellular adhesion molecule 1 (ICAM-1) (**Figure 4-16**). Thus, AvCystatin-MΦ express a surface marker pattern which, in this specific combination, has not yet been described for any other murine macrophage population.

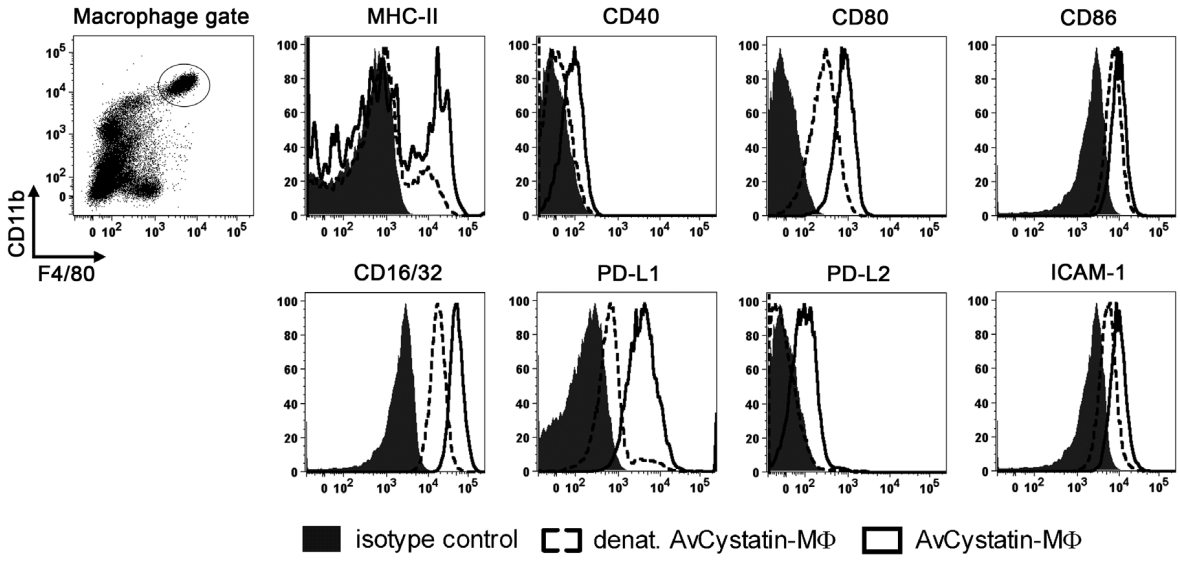

Figure 4-16. Surface marker expression of AvCystatin-MΦ. PEC were isolated from the peritoneal cavity of mice 18-20 hours after intraperitoneal application of 20 µg AvCystatin or control treatment. AvCystatin-stimulated F4/80$^+$CD11b$^+$ peritoneal macrophages show an up-regulation of MHC-II, co-stimulatory molecules (CD40, CD80, CD86), FcγRIII/FcγRII (CD16/32), PD-L1, PD-L2 and ICAM-1. Data represent one of three independent experiments. Cells were pooled from 5-6 mice per group.

4.3.4 Analyses of the AvCystatin-MΦ suppressive mechanism

4.3.4.1 Specificity of IL-10 induction by AvCystatin-MΦ

Since macrophage treatment with AvCystatin induced a phenotype similar to murine M1, M2a and M2b macrophage activation, AvCystatin-MΦ were examined for their specific characteristics in inducing $CD4^+IL\text{-}10^+$ T cells. M1, M2a and M2b macrophages were *in vitro* generated through stimulation of murine peritoneal macrophages with 100 U/ml IFN-γ and 10 ng/ml LPS (M1-MΦ) for 18-20 hours, through pre-treatment with 20 ng/ml IL-21 for 6 hours and subsequent incubation with 20 ng/ml IL-4/IL-13 for 18-20 hours (M2a-MΦ) or by stimulation with 150 µg ovalbumin immune complexes (OVA-IC) in combination with 10 ng/ml LPS for 18-20 hours (M2b-MΦ). Successful generation of the respective macrophage populations was determined by quantitative real-time PCR for the expression of selected M1 (iNOS, IL-12/23p40), M2a (ARG-1, RELM-α, Ym1) and M2b (IL-10, SPHK-1, LIGHT) activation markers (**Figure 4-17, A**). The ability of M1, M2a and M2b macrophages to induce IL-10 in $CD4^+$ T cells was evaluated by using the established co-culture assay of macrophages, ovalbumin-peptide-treated dendritic cells and ovalbumin-specific $CD4^+$ T cells. This was compared to the IL-10-inducing capacity of *in vitro*- and *in vivo*-stimulated AvCystatin-MΦ. M2b but not M1 or M2a macrophages showed increased IL-10 production 18-20 hours after stimulation with the respective stimulus. However, IL-10 levels in the culture supernatant of M2b macrophages incubated with ovalbumin-peptide-treated dendritic cells and $CD4^+$ T cells did not significantly differ from IL-10 levels produced by M2b macrophages alone (**Figure 4-17, B**). AvCystatin-MΦ showed no IL-10 self-production 18-20 hours after treatment with AvCystatin but significantly elevated levels of IL-10 were detected in culture supernatants of *in vivo*-stimulated AvCystatin-MΦ incubated with ovalbumin-peptide-treated dendritic cells and $CD4^+$ T cells. *In vitro*-generated AvCystatin-MΦ, however, lacked the ability to induce $CD4^+IL\text{-}10^+$ T cells (**Figure 4-17, B**).

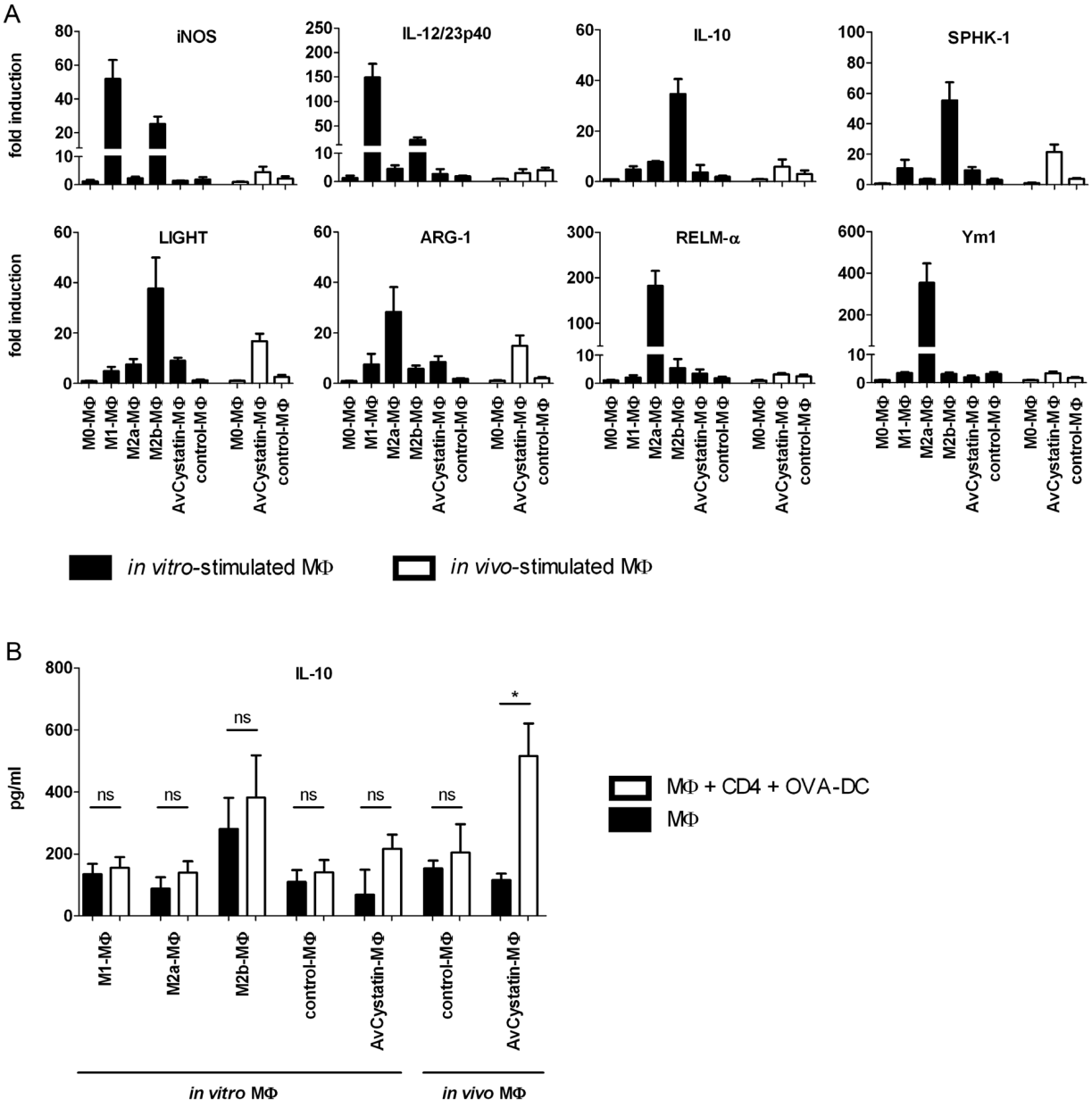

Figure 4-17. Macrophage-specific IL-10 induction. M1 macrophages (M1-MΦ), M2a macrophages (M2a-MΦ), M2b macrophages (M2b-MΦ), AvCystatin-stimulated macrophages (AvCystatin-MΦ) and denatured AvCystatin-treated macrophages (control-MΦ) were generated and *in vitro* evaluated for their ability to induce IL-10 in $CD4^+$ T cells. (A) Determination of macrophage activation by quantitative real-time PCR analysis for macrophage-specific marker expression 18-20 hours after treatment with the respective stimuli (M1-MΦ: 100 U/ml IFN-γ and 10 ng/ml LPS; M2a-MΦ: 20 ng/ml IL-21 and 20 ng/ml IL-4/IL-13; M2b-MΦ: 150 µg OVA-IC and 10 ng/ml LPS; *in vivo*-generated AvCystatin-MΦ: 20 µg AvCystatin/mouse; *in vitro*-generated AvCystatin-MΦ: 0.5 µM AvCystatin; control-MΦ were generated in an analogous manner by using denatured AvCystatin). Normalized data (mean ± SEM) of one representative experiment is shown and expressed as fold induction compared to untreated macrophages (M0). Macrophages of 5 mice per treatment were pooled and gene expression analysed in duplicate values. (B) Macrophage subpopulations were cultured alone (black bars) or with ovalbumin-specific $CD4^+$ T cells and ovalbumin-peptide-treated dendritic cells (OVA-DC) (white bars). Culture supernatants were analysed for IL-10 by ELISA. Pooled data of 3 independent experiments is presented as mean ± SEM. *, $P < 0.05$.

4.3.4.2 Role of macrophage markers for the induction of $CD4^+IL\text{-}10^+$ T cells

SPHK-1, ARG-1, PD-L1 and PD-L2 have formerly been reported to directly or indirectly interfere with $CD4^+$ T cell responses (Wang et al., 2005; Pesce et al., 2009 b; Francisco et al., 2009). To address whether these marker genes are involved in the induction of $CD4^+IL\text{-}10^+$ T cells, either chemical inhibitors and blocking antibodies for ARG-1, PD-L1, PD-L2 or antagonists for target receptors of AvCystatin-MΦ-secreted factors (S1P) were used. AvCystatin-MΦ were pre-treated with different concentrations of the ARG-1 inhibitor S-(2-boronoethyl)-l-cysteine (BEC) (**Figure 4-18, A**) or with anti-PD-L1 and anti-PD-L2 antibodies in a dose-dependent manner for one hour (**Figure 4-18, E**) and thereafter cultured with ovalbumin-petide-treated dendritic cells (OVA-DC) and ovalbumin-specific $CD4^+$ T cells. The efficiency of ARG-1 inhibition by BEC was determined through measuring urea production by M2a macrophages in the absence of BEC or in the presence of different BEC concentrations (**Figure 4-18, B**). Since SPHK-1 converts sphingosine to sphingosine-1-phosphate (S1P) which can bind to sphingosine 1 phosphate receptors (S1PR) on T cells, ovalbumin-specific $CD4^+$ T cells were pre-treated with the S1PR1 antagonist VPC23019 for one hour in a dose-dependent manner and further incubated with AvCystatin- or control-treated macrophages and ovalbumin-peptide-treated dendritic cells (**Figure 4-18, C**). In addition, co-cultures of ovalbumin-peptide-treated dendritic cells and ovalbumin-specific $CD4^+$ T cells were supplemented with various concentrations of S1P to analyse whether addition of the lysosphingophospholipid itself induces IL-10 production in ovalbumin-specific $CD4^+$ T cells (**Figure 4-18, D**). None of the inhibitors, blocking antibodies or antagonists significantly reduced IL-10 levels in the supernatants of co-cultured AvCystatin-MΦ, ovalbumin-peptide-treated dendritic cells and ovalbumin-specific $CD4^+$ T cells (**Figure 4-18, A, C** and **E**). Moreover, supplementation of $CD4^+$ T cell and dendritic cells co-cultures with S1P did not increase IL-10 levels in the culture supernatants (**Figure 4-18, D**). Together, these findings argue for an additional, as yet undefined factor secreted by AvCystatin-MΦ that conveys the IL-10-inducing capacity.

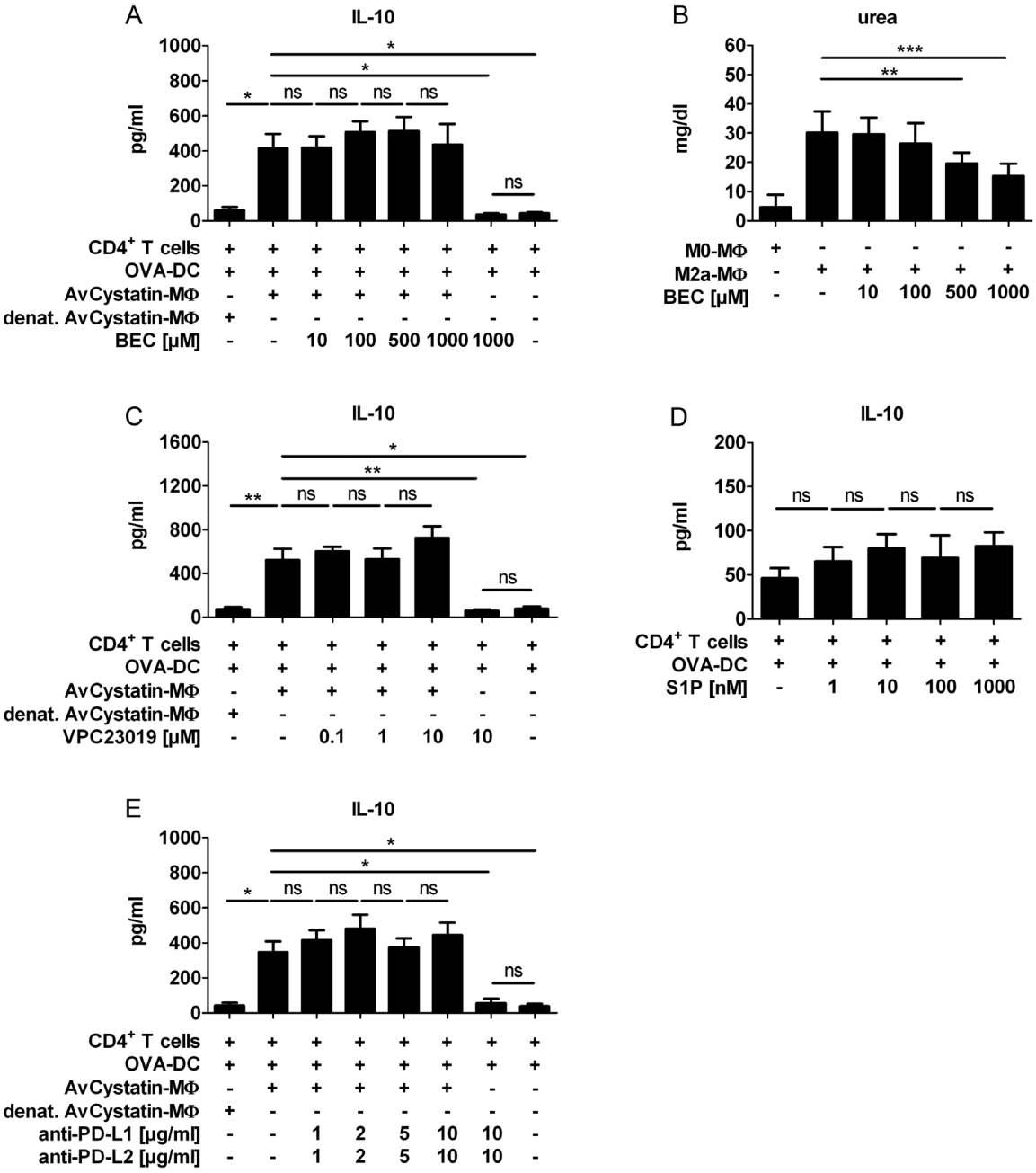

Figure 4-18. Role of specific AvCystatin-MΦ markers for the induction of IL-10 in T cells. (A) Macrophages were pre-treated for one hour with different concentrations of the ARG-1 inhibitor BEC and thereafter incubated with ovalbumin-specific $CD4^+$ T cells and ovalbumin-peptide-treated dendritic cells (OVA-DC). (B) Validation of BEC activity was determined by measuring urea concentrations in the supernatants of M2a macrophage cultures after incubation with different concentrations of the inhibitor for 18-20 hours. (C) Ovalbumin-specific $CD4^+$ T cells were pre-incubated for one hour with the S1PR1 antagonist VPC23019 and subsequently incubated with AvCystatin- or control-treated macrophages and ovalbumin-peptide-treated dendritic cells. (D) Co-cultures of ovalbumin-specific $CD4^+$ T cells and ovalbumin-peptide-treated dendritic cells were supplemented with various concentrations of S1P. (E) Macrophages were pre-treated with anti-PD-L1 and anti-PD-L2 for one hour in a dose-dependent manner and further incubated with ovalbumin-specific $CD4^+$ T cells and ovalbumin-peptide-treated dendritic cells. Data for one representative of 2-3 independent experiments is presented as mean ± SEM in A, C, D and E. Pooled data from 3 experiments is shown in B. For each experiment culture assays were performed with pooled macrophages of 5-6 animals per treatment, purified splenic $CD4^+$ T cells of 3 DO11.10 mice and bone marrow-derived dendritic cells of one animal. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4.4 Proposed model of AvCystatin-induced macrophage activities

In order to summarize the experimental findings of the present thesis, the following model of AvCystatin-induced macrophage activities is suggested (**Figure 4-19**).

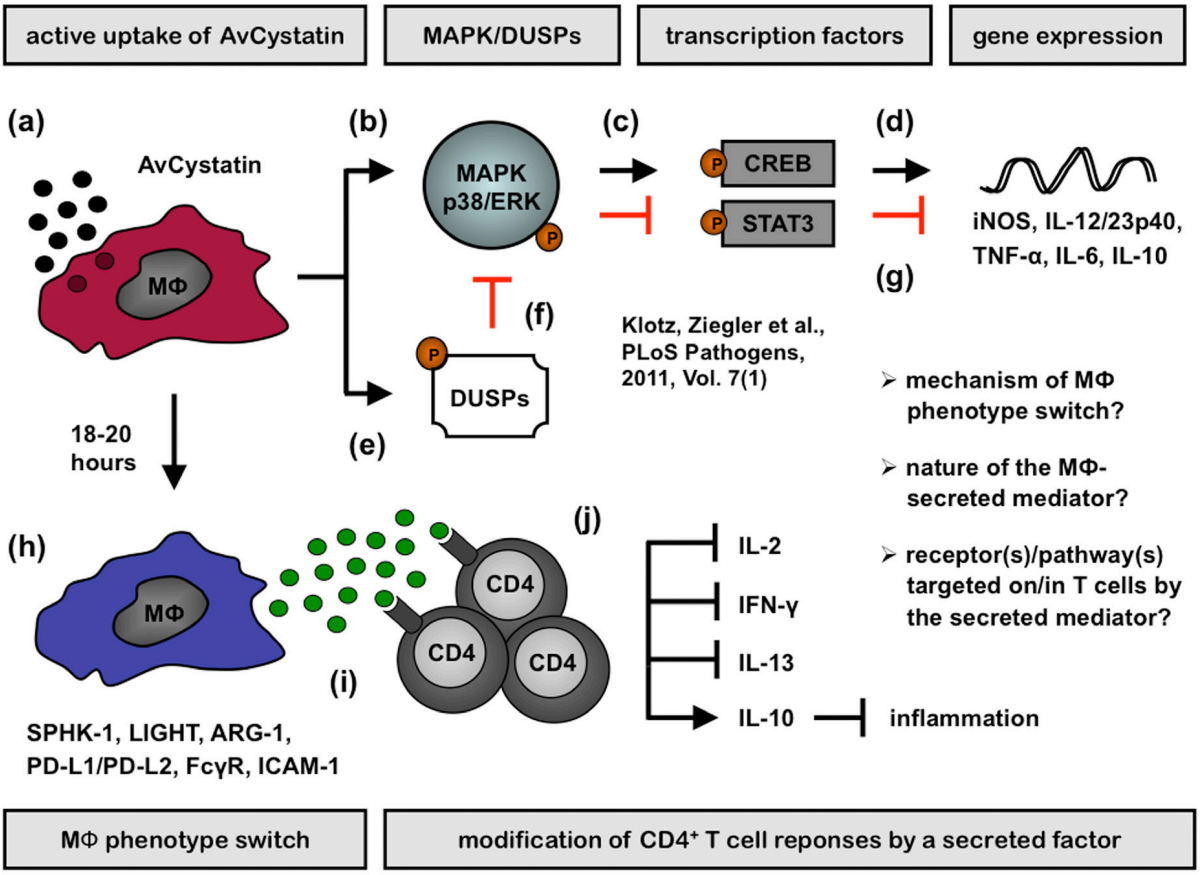

Figure 4-19. Modulation of murine macrophage activities by AvCystatin. Treatment of peritoneal macrophages with AvCystatin leads to an active endocytotic uptake of the immunomodulator into the endolysosomal compartment (a) and induces subsequent phosphorylation of MAPK p38 and ERK (b). The activation of transcription factors CREB and STAT3 by MAPK (c) gives rise to the expression of pro- and anti-inflammatory marker genes (d). In parallel to MAPK, dual specificity phosphatases (DUSPs) are expressed (e) which act as negative regulators on MAPK (f). As a result of DUSP-mediated dephosphorylation of p38 and ERK, the early gene expression is down-modulated (g). Macrophages show an M2a/M2b hybrid phenotype 18-20 hours post stimulation with the immunomodulator, characterized by the expression of SPHK-1, LIGHT and ARG-1 (h). Adoptive transfer of this phenotype mitigates intestinal inflammation and symptoms of ovalbumin-induced airway hyperreactivity in mice. The amelioration of airway inflammation is associated with the secretion of a mediator (i) that induces $CD4^+$ T cells which produce high amounts of IL-10 but reduced levels of IL-2, IFN-γ and IL-13 (j).

5 DISCUSSION

The immunomodulatory potential of parasitic worms and the suppression of unrelated pathology as a bystander effect of helminth infection has led to an increased interest in the interplay between parasite-induced evasion mechanisms and the immune system of their mammalian hosts. The present investigation studied the effects of the helminth-derived immunomodulatory protein AvCystatin on murine peritoneal macrophages and aimed to examine the therapeutic potential of AvCystatin-MΦ in two murine disease models.

5.1 Part I: Signalling events in AvCystatin-MΦ

In previous studies, AvCystatin was shown to affect the cytokine response of mouse macrophages by inducing an anti-inflammatory IL-10-secreting phenotype (Schierack et al., 2003). Additional investigations demonstrated IL-10 to be the key mediator of the AvCystatin-mediated immunosuppression in a model of ovalbumin-induced airway hyperreactivity. Administration of anti-IL-10 antibodies prior to allergen challenge ablated the protective AvCystatin effect in mice and the depletion of macrophages resulted in abrogated IL-10 expression. These results suggested that AvCystatin ameliorates inflammatory responses through the induction of IL-10-producing macrophages (Schnoeller et al., 2008). To date, little is known about intracellular pathways and signalling events which are target of and triggered by AvCystatin. Part I of the present investigation thus aimed to analyse signalling events involed in the IL-10 induction of AvCystatin-MΦ.

5.1.1 Activation of MAPK affects IL-10 production in macrophages

The importance of MAPK activation for cytokine production has been investigated in and demonstrated through different studies (Saccani et al., 2002; Mathur et al., 2004). To assess whether MAPK are involved in the AvCystatin-mediated induction of IL-10 in macrophages, the influence of specific MAPK inhibitors on IL-10 production in murine peritoneal macrophages was examined. In mammalian cells three important MAPK pathways are described: the p38, ERK and JNK pathway (Johnson and Lapadat et al., 2002). These MAPK are activated through phosphorylation of their tyrosine and threonine residues and target a large number of downstream elements such as kinases, transcription factors or proteins which control the stability and translation of mRNA (Lang et al., 2006). In the present study, inhibition of both p38 and ERK clearly affected IL-10 production by AvCystatin-MΦ in a dose- and tyrosine kinase-dependent manner whereas blockade of JNK had no significant effect on IL-10 production. Suppressed IL-10 levels in culture supernatants after PI3K inhibition further

argued for an involvement of PI3K-AKT in AvCystatin-induced IL-10 expression. MAPK activation through phosphorylation of tyrosine and threonine residues alters protein conformation and substrate accessibility (Cobb and Goldsmith, 2000). Therefore, additional western blot analysis was performed that supported the experimental outcome of the inhibitor study by confirming AvCystatin-induced phosphorylation of p38 and ERK. Surprisingly, phosphorylation of the PI3K downstream target AKT was not detectable although blockade of PI3K significantly inhibited IL-10 production. Together, these observations suggest that activation of p38 and ERK leads to IL-10 production in macrophages and endogenous phospho-AKT levels might be enough for sufficient cytokine production after treatment with AvCystatin.

The AvCystatin-specific activation of MAPK and its positive correlation with IL-10 production in macrophages is in line with findings of Lucas et al. (2005) who determined the mechanism of IL-10 induction in regulatory M2b macrophages. The authors found up-regulation of IL-10 to be associated with enhanced activation of p38 and ERK. Increased ERK activity led to histone modifications at the IL-10 locus resulting in higher IL-10 promotor accessibility to p38-activated transcription factors specificity protein 1 (Sp1) and signal transducer and activator of transcription (STAT) 3. Phosphorylation of transcription factors is an important feature of activated MAPK for the induction of gene transcription (Davis, 1993). Activation of transcription factor cAMP response element-binding protein (CREB) was shown to correlate with increased promotor binding and IL-10 expression in macrophages (Martin et al., 2005). In addition, CCAAT/enhancer-binding protein (C/EBP), nuclear factor-kappa B (NFkappaB), interferon regulatory factor-1 (IRF-1) and musculoaponeurotic fibrosarcoma (Maf) have been determined as important regulators of IL-10 production in macrophages (Liu et al., 2003; Cao et al., 2006; Ziegler-Heitbrock et al., 2003; Cao et al., 2005). In additional studies, which were not directly part of the present thesis, AvCystatin was found to induce MAPK-regulated phosphorylation of transcription factors CREB and STAT3 in murine peritoneal macrophages (Klotz et al., 2011 b). It remains to be analysed whether treatment of macropages with AvCystatin additionally promotes histone phosphorylation and IL-10 promoter accessibility similar to what has been described by Lucas et al. (2005).

The activation of MAPK is not a mechanism specific to AvCystatin. Other helminth-derived molecules have been shown to exploit MAPK signalling in order to modulate APC maturation (reviewed in Carvalho et al., 2009). ES-62 of the rodent nematode *Acanthocheilonema viteae* activates ERK but suppresses p38 phosphorylation thereby directing the phenotype of dendritic cells towards low IL-12 production (Goodridge et al., 2005 b). Similar to ES-62, the immunomodulatory glycan LNFPIII of the blood fluke *Schistosoma mansoni* exerts Th2-

promoting activity on dendritic cells by preferentially activating ERK (Thomas et al., 2003). This demonstrates that helminth parasites target and subvert well-established signalling pathways in host cells to modify their cytokine response.

5.1.2 Dual-specificity phosphatases regulate MAPK activity

Stimulation of macrophages with AvCystatin induced transient activation of both p38 and ERK. Analysis of IL-10 transcript further revealed early induction of IL-10 followed by a subsequent down-regulation to background levels. To explain this transient activation and expression, a negative feedback mechanism on MAPK was hypothesized. Inactivation of MAPK activity can be performed by serine-threonine phosphatases, tyrosine phosphatases or dual-specificity phosphatases (DUSPs) (Lang et al., 2006). DUSPs are particularly effective regulators of MAPK since they dephosphorylate both tyrosine and threonine residues (Liu et al., 2007). To date, approximately 11 of 30 DUSPs have been shown to contain a MAPK binding domain in addition to their DUSP domain and thereby exert inhibitory activity on MAPK (Lang et al., 2006). To elucidate whether DUSPs are responsible for transient phosphorylation of p38 and ERK, the AvCystatin-specific expression of selected DUSPs which have been reported to regulate p38 and ERK activity in macrophages *in vitro* and *in vivo* was determined. Real-time PCR analysis of AvCystatin-MΦ revealed up-regulation of DUSP-1, DUSP-2 and lower amounts of DUSP-5 *in vitro*. *In vivo*-stimulated macrophages showed prominent expression of DUSP-1 and moderate transcript levels of DUSP-2. DUSP expression was detectable as early as 30 to 60 minutes after treatment with AvCystatin. DUSPs can be divided into phosphatases which are predominantely localized in the nucleus and encoded by immediate-early genes and phosphatases which are not encoded by immediate-early genes and primarily reside in the cytoplasm or both the cytoplasm and the nucleus (Liu et al., 2007). The affiliation of DUSP-1, DUSP-2 and DUSP-5 to phosphatases encoded by immediate-early genes might explain their early expression after stimulation with AvCystatin. DUSPs that belong to this group of phosphatases are normally induced by the same stimuli that lead to MAPK activation, e.g. stress signals or growth factors (Keyse, 2000).

Phosphorylation is not essential for DUSP activation but mediates stabilization of DUSPs by increasing their half-life. The accumulation of DUSPs in consequence leads to higher DUSP activity (Brondello et al., 1999). To elucidate a possible stabilization effect on DUSPs, the phosphorylation kinetics of DUSP-1 was investigated in AvCystatin-MΦ by western blot analysis. Increased DUSP-1 phosphorylation was detected after 60 minutes of stimulation suggesting that the phosphatase is getting stabilizied through treatment with AvCystatin. It is

possible that DUSP-1 stabilization might be an effect of the early IL-10 production by AvCystatin-MΦ since an investigation by Hammer et al. (2005) showed that IL-10 can enhance and prolong the expression of DUSP-1. By using DUSP-1-deficient mice, DUSP-1 was demonstrated to control phosphorylation of MAPK and IL-10 expression following AvCystatin treatment. Wildtype animals showed transient expression of IL-10 after stimulation with the immunomodulator whereas DUSP-1-deficient mice revealed increased and sustained IL-10 expression (**chapter 4.2.4**) but reduced levels of IL-12/23p40 (data not shown). This result is substantiated by studies showing that DUSP-1-deficient mice produce increased amounts of IL-10, TNF-α and IL-6 but reduced levels of IFN-γ and IL-12p40 in response to TLR stimulation (reviewed in Lang et al., 2006). Western blotting confirmed the substrate preference of DUSP-1 for p38 since treatment with AvCystatin led to an accumulation of phospho-p38 and slightly reduced levels of phospho ERK in DUSP-1-deficient mice.

5.1.3 Possible involvement of receptors in AvCystatin-mediated signalling

AvCystatin was actively taken up into the endolysosomal compartment of peritoneal macrophages. The activation of MAPK following AvCystatin treatment, however, might be indicative for an additional, receptor-mediated mechanism. So far, no specific receptors have been identified for cystatins. Thus, it is still unclear whether AvCystatin effects result from receptor binding or from inhibitory effects on cysteine proteases (Klotz et al., 2011 b). Human cystatin C was shown to antagonize TGF-β signalling through interaction with the TGF-β receptor on mammary tumor cells (Sokol et al., 2005). By using a TGF-β reporter system described in Tesseur et al. (2006), AvCystatin was not determined to interfere with TGF-β signalling (data not shown). Another feasible scenario is that AvCystatin interacts with PRRs such as TLRs or C-type lectin receptors. Several helminth molecules have been described to induce anti-inflammatory responses by interference with PRRs (reviewed in Harnett and Harnett, 2010). The aminopeptidase ES-62, for example, signals through TLR-4 in a manner which is different from LPS (Goodridge et al., 2005 a). Moreover, products from schistosomes (e.g. lacto-N-fucopentaose, lysophosphatidylserine or phosphatidylserine) exert anti-inflammatory effects through the interaction with TLRs (van der Kleij et al., 2002; Harn et al., 2009; van Riet et al., 2009). In studies from our own group, *Acanthocheilonema viteae*-derived tropomyosin was found to up-regulate and bind TLR-2 on mouse macrophages (unpublished data). TLR-3, TLR-7, TLR-8, TLR-9 and TLR-13 are intracellularly expressed and described to act within the endosomal compartment (reviewed in Blasius and Beutler, 2012). Since AvCystatin is taken up into the endolysosomal compartment, the immunomodulator could theoretically bind to intracellular TLRs. It might be

astonishing that helminth parasites convey anti-inflammatory effects via TLRs however my be plausible if one takes into account that subversion of TLR signalling by filarial nematodes supports the generation of immature APCs which produce fewer pro-inflammatory cytokines (Harnett and Harnett, 2010). The precise mechanisms how helminths modulate TLR responses are not entirely known. In a study by Donnelly et al. (2010), two helminth cysteine proteases, *Fasciola hepatica* cathepsin L1 (FheCL1) and *Schistosoma mansoni* cathepsin B1 protease (SmCB1), were shown to successfully protect mice against the development of severe inflammation in response to TLR agonists through the suppression of TRIF-dependent macrophage activation. In the respective study, the protease-specific inhibition of TLR signalling was mediated through degradation of TLR-3 within the endosome (Donnelly et al., 2010). Park et al. (2008) showed that cathepsin activity is necessary for TLR-9 responses. The investigators blocked cathepsin activity by using the cysteine protease inhibitor benzyloxycarbonyl-Phe-Ala-fluoromethylketone (z-Phe-Ala-fmk) and thereby inhibited TNF-α production in RAW cells (a murine leukaemic monocyte-macrophage cell line) in response to CpG oligodeoxynucleotides but not LPS. Thus, it is likely that helminth-secreted cysteine protease inhibitors such as AvCystatin interfere with TLR-9 responses in the endolysosomal compartment. Moreover, accessory PRRs, e.g. C-type lectins, might be recruited and crosstalk with TLRs in order to inhibit or synergistically enhance signals (reviewed in Harnett and Harnett, 2010). C-type lectins such as the mannan-binding lectin (MBL), macrophage galactose-type lectin (MGL) or dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), have been shown to act as targets for SEA or different glycans (e.g. Lewis^x antigen or α/β GalNAc) of *Schistosoma mansoni* (reviewed in Johnston et al., 2009). At present, the most promising receptor candidates for AvCystatin are Fcγ receptors. AvCystatin-MΦ share remarkable similarities with M2b macrophages which are derived through crosslinking of Fcγ receptors by immune complexes in the presence of a pro-inflammatory co-stimulus. Moreover, AvCystatin targets signalling events in murine macrophages similar to those described for M2b macrophages (Lucas et al., 2005) and up-regulates the expression of Fcγ receptors on these cells. *In vivo*, AvCystatin induces high levels of IgG1 (data not shown) which is known to bind with high affinity to Fcγ receptors. These findings hint towards a possible involvement of Fcγ receptors in AvCystatin-mediated signalling.

Together, the results of part I show for the first time that the helminth immuomodulator AvCystatin exploits activating and deactivating pathways of MAPK in murine macrophages thereby regulating important biological functions such as cytokine production.

5.2 Part II: Functional and molecular characteristics of AvCystatin-MΦ

In part I, AvCystatin was determined to interfere with signalling events leading to IL-10 production in peritoneal macrophages. The aim of part II was to examine the potential of AvCystatin-MΦ in transferring protection against aberrant inflammation independent of the immunomodulator`s presence and to gain more insights into the macrophage phenotype and the macrophage-mediated mechanism of immunosuppression.

5.2.1 AvCystatin-MΦ ameliorate ovalbumin-induced airway inflammation

The model of ovalbumin-induced airway hyperreactivity in mice reflects important symptoms of asthma. After antigen-sensitization and -challenge, animals develop airway inflammation characterized by increased levels of Th2 cytokines, production of antigen-specific IgE, airway eosinophilia and goblet cell hyperplasia (Schröder and Maurer, 2007). The present study shows that single transfer of AvCystatin-MΦ is sufficient to abrogate pathophysiological hallmarks of ovalbumin-induced airway hyperreactivity. Suppression of allergic inflammation either involves counteracting mechanisms such as driving the immune response towards a Th1 phenotype or results from unspecific suppression of immune responses and leucocyte activity (Peters-Golden, 2004). Adoptive transfer of AvCystatin-MΦ did not instruct a counter-regulatory Th1 response in recipient mice (data not shown) but correlated with high levels of local and systemic IL-10 through induction of $CD4^+IL\text{-}10^+Foxp3^-$ T cells. Previous studies demonstrated the necessity of IL-10 for the AvCystatin-mediated suppression of ovalbumin-induced airway hyperreactivity in mice since *in vivo* blockade of the IL-10 receptor reversed protection (Schnoeller et al., 2008). IL-10 has been shown to exert suppression on APCs, eosinophils, mast cells, basophils and T cells thus representing an attractive candidate to control allergic diseases (Hawrylowicz and O´Garra, 2005; Saraiva and O´Garra, 2010). Compared to increased IL-10 production, AvCystatin-MΦ-induced $CD4^+Foxp3^-$ T cells showed a reduced capacity to produce other cytokines. Collectively, these results demonstrate that AvCystatin-MΦ modulate the antigen-specific cytokine response of $CD4^+$ T cells and thereby indirectly abrogate the main features of ovalbumin-induced airway hyperreactivity. However, the induction of IL-10-producing $CD4^+$ T cells is not a feature specific to AvCystatin-MΦ. A similar effect has been described by Atochina et al. (2008). In the respective study, murine peritoneal macrophages were treated *in vivo* with LNFPIII, a secreted glycan of the blood fluke *Schistosoma mansoni*. Macrophages were isolated after 20 hours and exhibited an alternative activation status through up-regulation of ARG-1 and Ym-1. These macrophages were pulsed *ex vivo* with ovalbumin-peptide and adoptively transferred into the peritoneum of healthy DO11.10 mice. Splenocytes of recipient animals were prepared 5-6 days post transfer and cultured for 72 hours in the presence of ovalbumin-

peptide. Thereby, LNFPIII- but not control-treated macrophages significantly increased levels of IL-10 but also induced the production of IL-13 in $CD4^+$ T cells. Although not explicitly shown, the authors speculated that this effect could be mediated through macrophage soluble factors (Atochina et al., 2008).

Th2 cytokines are key factors for the development of asthma pathogenesis. IL-5 plays an outstanding role for eosinophilopoesis, the recruitment of eosinophils to the site of inflammation, their degranulation and survival (reviewed in O´Byrne et al., 2004). IL-4 is a cytokine with pleiotropic effects on different cells and important for the initial phase of allergic inflammation. It increases Fcε receptor expression on B cells, promotes isotype switching to IgE (Bossie et al., 1987; Hudak et al., 1987), stimulates Th2 cell differentiation (Spellberg and Edwards, 2001), mediates tissue homing of inflammatory cells (Romagnani, 2004) and triggers mast cell growth and maturation (Paul, 1989). IL-13 has been shown to mediate airway hyperresponsiveness and mucus secretion (Wills-Karp et al., 1998; Walter et al., 2001). IL-4 and IL-13 both recruit eosinophils, macrophages, monocytes and $CD4^+$ T cells from the bloodstream by inducing expression of adhesion molecules in the airway epithelium (Bochner et al., 1995) and chemokines that regulate chemotaxis of inflammatory cells (Schuh et al., 2003; Zimmermann et al., 2003). Modulation of local and systemic Th2 responses after transfer of AvCystatin-MΦ was associated with the induction of IL-10-producing $CD4^+$ T cells. Since IL-10 inhibits Th2 cytokine production by the suppression of CD28-dependent signalling in T cells (Schandene et al., 1994; Akdis et al., 2000; Akdis and Blaser 2001), it is possible that the AvCystatin-MΦ-induced IL-10 acts back on T cells in an autocrine manner thereby suppressing cytokine production. To test this hypothesis, ovalbumin-specific $CD4^+$ T cells were incubated *in vitro* with an anti-IL-10 receptor antibody and thereafter co-cultured with ovalbumin-peptide-treated dendritic cells and AvCystatin-MΦ. Blocking IL-10 signalling in T cells did not restore the AvCystatin-MΦ-mediated suppression of IL-13 but significantly reversed the inhibition of IL-2. These findings exclude an autocrine IL-10 feedback to be responsible for the observed suppression of Th2 cytokines in $CD4^+$ T cells but indicate that IL-10 might suppress T cell proliferation through the inhibition of IL-2 production and thereby indirectly limit Th2 cytokine production. The main source of Th2 cytokines are $CD4^+$ Th2 T cells. Eosinophils, basophils and mast cells, however, mobilize IL-4 and IL-13 transcripts for instantaneous translation and can therefore act as early sources of Th2 cytokines (Gessner et al., 2005). This leads to the question whether such cells can compensate the AvCystatin-MΦ-mediated Th2 cytokine suppression. It has been shown that intranasal allergen challenge is important for the recruitment of basophils, mast cells and eosinophils to the airways (reviewed in Kim et al., 2010). AvCystatin-MΦ were transferred 4 days prior to intranasal provocation with ovalbumin. Therefore, it is possible that AvCystatin-MΦ interfere

with the recruitment of inflammatory cells, e.g. through the suppression of respective chemokines such as eotaxins, and thereby prevent the onset of inflammaton. With respect to continuing studies, it might be interesting to address whether transfer of AvCystatin-MΦ is still protective after intranasal challenge.

Allergic asthma is correlated with elevated levels of antigen-specific IgE (Holgate, 2008) that becomes attached to Fcε receptors on mast cells, basophils and eosinophils through a specific domain of the IgE constant region (Presta et al., 1994; Platts-Mills, 2001 b). The involvement of IgE in the development of asthma has been demonstrated by showing that passive transfer of antigen-specific IgE antibodies is sufficient to induce cutaneous hypersensitivity and increased airway responsiveness in unsensitized recipient mice (Oshiba et al., 1996). Moreover, injection of plasma from atopic individuals caused positive allergen skin test reactions in nonatopic individuals (reviewed in Platts-Mills, 2001 b). IgE itself is supposed to promote the up-regulation of high-affinity Fcε receptors on effector cells which could explain its high biological activity even when present in low concentrations (Yamaguchi et al., 1997). Crosslinking of two adjacent IgE molecules by antigen leads to the release of histamine, leukotrienes, prostaglandins, cytokines and chemokines (reviewed in Barrett and Austen, 2009). These mediators are responsible for immediate and late allergic reactions as they increase vascular permeability and promote the up-regulation of adhesion molecules for lymphocytes and other effector cells (reviewed in Platts-Mills, 2001 b). In the present study, transfer of AvCystatin-MΦ suppressed total- and antigen-specific IgE levels in recipient mice. IL-4 and IL-13 are crucial for both isotype switching by B cells and the secretion of allergen-specific IgE by plasma cells. Since AvCystatin-MΦ were transferred into already sensitized mice, it is unlikely that the macrophages prevent isotype switching to IgE, however through modulation of T cell responses and the resulting suppression of IL-4 and IL-13 they may have dampened the release of IgE by plasma cells in response to intranasal provocation which, in turn, limits effector cell activation and degranulation. The latter scenario has experimentally been demonstrated in previous studies by showing that the sensitization of basophils with sera of ovalbumin/AvCystatin-treated mice induced less degranulation compared to basophil sensitization with sera of control animals (Schnoeller et al., 2008).

In addition, transfer of AvCystatin-MΦ significantly reduced airway eosinophilia as detected by cell counts for eosinophils in the BAL-fluid. Eosinophils are major players in asthma. They produce Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-5, IL-13) cytokines, act as APCs and modulate other cells, e.g. dendritic cells, $CD4^+$ T cells, B cells, mast cells, basophils and neutrophils (reviewed in Akuthota et al., 2008). IL-10 was reported to interfere with LPS-induced eosinophil survival and cytokine release in a dose-dependent manner (Takanaski et al.,

1994) suggesting that the AvCystatin-MΦ-induced IL-10 production by T cells might hamper eosinophil activity. Another possibility is that AvCystatin-MΦ interfere with eosinophil recruitment. In the present study, it was found that reduced eosinophil numbers in the BAL-fluid were associated with suppressed expression of eotaxin-1 (CCL11), eotaxin-2 (CCL24), CCR3 and MCP-5 (CCL12) in lung homogenates. In the lung, eotaxin-1, eotaxin-2 and MCP-5 are commonly produced by lung fibroblasts and epithelial cells. AvCystatin-MΦ could not be detected in the lungs of recipient mice. Thus, it seems unlikely that the suppression of eotaxin production is mediated through cell-cell interaction. However, AvCystatin-MΦ were tracked in the peribronchial lymph nodes and could probably secrete mediators which are transported to the lungs and either silence eotaxin expression by the respective cells or actively cleave eotaxins. The latter mechanism has been described for hookworm metalloproteases *in vivo* and *in vitro* (Culley et al., 2000). Although the inhibition of eotaxin activity through macrophage-secreted metalloproteases could explain the reduced eosinophil numbers in the BAL-fluid after transfer of AvCystatin-MΦ, it does not explain the observed suppression of eotaxin transcript levels in lung homogenates. Thus, it is yet unclear whether metalloproteases are involved in AvCystatin-MΦ-mediated suppression of eotaxins. Alternatively, AvCystatin-MΦ might modulate $CD4^+$ T cells in the spleen or PBLNs which migrate to the lungs in response to allergen-exposure and suppress eotaxin production by local fibroblasts or epithelial cells. Eotaxin activity is mediated through the eotaxin receptor CCR3 which is primarily expressed on eosinophils (Lukacs et al., 2003; Pope et al., 2005). The detection of lowered CCR3 transcript levels in homogenized lung tissue after treatment with AvCystatin-MΦ nicely reflects the reduced eosinophil number in the BAL-fluid. MCP-5 is important for the early phase of airway inflammation. It is chemotactic for eosinophils and other leukocytes and promotes their migration through the lung interstitium (Gonzalo et al., 1998; Jia et al., 1996). Thus, AvCystatin-MΦ might not only suppress chemoattraction of eosinophils but may also inhibit eosinophil migration through suppression of MCP-5.

Hypersecretion of mucus contributes to airway obstruction and hyperresponsiveness. Under healthy conditions, mucociliary clearance protects the airways against infections and enables normal lung functions. Inhaled pollutants and particles are trapped in a thin liquid layer, transported to the tip of the cilia and removed by cilia beating. In chronic asthma, increased mucus production leads to plugging of the airways, impaired airflow, induces cough and affects ciliary clearance functions (reviewied in Rogers, 2004). IL-13 was shown to mediate goblet cell hyperplasia and to hold important functions for the induction of mucus in the airways (Kuperman et al., 2002; Whittaker et al., 2002; Izuhara et al., 2009). The suppression of IL-13 in $CD4^+$ T cells after contact with AvCystatin-MΦ-secreted factors possibly explains the reduced mucus production in recipient animals. In a study by Yang et

al. (2003 b), IL-5 and eotaxin-2 were shown to cooperatively promote the production of IL-13 through recruitment of eosinophils to the lung of mice in an experimental model of eotaxin-2-induced airway eosinophilia. Different cell populations of the BAL-fluid were examined for their capacity to produce IL-13. Thereby, eosinophils but not lymphocytes were determined as the primary source of IL-13. In the present study, suppression of IL-13 and mucus production could thus also depict a consequence of the AvCystatin-MΦ-mediated suppression of IL-5 and/or eotaxins.

To evaluate whether the immunosuppression by AvCystatin-MΦ is cell-mediated and not a "carry-over" effect of AvCystatin, splenocytes of recipient mice treated with either AvCystatin- or control-stimulated macrophages were *in vitro* restimulated with 10 µg/ml AvCystatin for 96 hours. Analyses of culture supernatants via ELISA revealed no AvCystatin-specific cytokine response (data not shown). In parallel, the fate of iodinated AvCystatin was determined by cooperation partners after intravenous and intraperitoneal application into mice. Thereby, rapid urinary excretion of the protein for both application modes was detected after 18-20 hours (data not shown). This shows that AvCystatin modulates macrophages during a short time frame and that the induced macrophage population is capable of efficiently ameliorating ovalbumin-induced airway hyperreactivity independent of the immunomodulator`s presence.

5.2.2 Macrophage tracking

To unravel the immunosuppressive properties of AvCystatin-MΦ in ovalbumin-induced airway inflammation, the localization of transferred macrophages was examined in different tissues of the recipient mice. CFSE-labelled macrophages were detected in the spleen (AvCystatin-MΦ: 194491 ± 27549 cells; control-treated MΦ: 94680 ± 10013 cells; mean ± SEM) and to a lower amount in the PBLNs (AvCystatin-MΦ: 3985 ± 2870; control-treated MΦ: 1824 ± 202; mean ± SEM) but not in the lungs or the BAL-fluid seven days after intravenous administration into recipient animals. Several studies support the idea that macrophages are long-lived cells. The half-life of murine alveolar macrophages supposedly ranges from 30 days to 2 years (Murphy et al., 2008; Tetley, 2002). *In vivo* tracking of IFN-γ-treated macrophages in a murine model of colitis showed that intravenously injected macrophages ($2.5x10^6$ cells/mouse) migrated to diverse organs and tissues such as the spleen, Peyer`s patches, mesenteric lymph nodes, colonic mucosa, lung, liver, bone marrow and cervical lymph nodes and were still detectable seven days after administration (Brem-Exner et al., 2008). In a murine model of adriamycin-induced nephropathy (AN), $1x10^6$ fluorescently labelled M2c-polarized or untreated splenic control macrophages (M0) were intravenously transferred into mice on day 5 after adriamycin treatment. Transferred M2c

macrophages were still detectable on day 28 in the kidneys and renal draining lymph nodes (RDLN) of recipient mice. Fewer macrophages were observed in animals treated with M0 macrophages (Cao et al., 2010). In a similar disease model, splenic macrophages were intravenously injected into severe combined immunodeficient (SCID) mice suffering from adriamycin-induced nephropathy after *in vitro* differentiation into M1 (LPS) or M2a (IL-4/IL-13) macrophages. In addition, untreated M0 macrophages were transferred. After injection, M0, M1 and M2a macrophages were similarly distributed in the kidneys, spleen and liver of recipient mice and maintained their phenotype for up to 4 weeks (Wang et al., 2007). By investigating trafficking patterns of C2D cell line macrophages (immature macrophages that show a MHC-II$^{-/-}$ and TLR-4$^{Lps\text{-}n}$ genotype), Potts et al. (2008) showed that intraperitoneally injected C2D macrophages were detectable as long as 2 months in the recipient mice. Together, these results suggest long-term survival of distinct macrophage populations and their suppressive properties. AvCystatin-MΦ were not detected in the lung or BAL-fluid but in PBLNs and the spleen. It is known that macrophages migrate to draining lymph nodes to prime T cell responses (Muller and Randolph, 1999; Randolph et al., 1999; Atochina et al., 2008). Therefore, AvCystatin-MΦ might exert an indirect suppression on airway inflammation through the modulation of T cell functions in the PBLNs or the spleen. Thus, further *in vivo* tracking studies are required to determine the stability and migration pattern of AvCystatin-MΦ in more detail and to examine specific compartments in which an interaction between AvCystatin-MΦ and T cells takes place such as the marginal zone of the spleen for example.

5.2.3 Relevance of other cell types in transferring AvCystatin effects

A number of studies show that specific B cell populations can mediate protection against allergic airway inflammation and EAE. In a study by Mangan et al. (2004), intraperitoneal injection of 0.5-1.0x10^7 splenic B cells of *Schistosoma mansoni*-infected mice protected recipient animals against systemic anaphylaxis through the secretion of IL-10. Wilson et al. (2010) isolated a population of CD19$^+$CD23^{high} B cells from the mesenteric lymph nodes of *Heligmosomoides polygyrus*-infected mice and showed that intravenous transfer of 4x10^6 B cells profoundly protects against Der p1-induced allergic inflammation and myelin oligodendrocyte glycoprotein peptide fragment 35-55 (MOG_{35-55} peptide)-induced EAE through an IL-10-independent mechanism. In another investigation, intraperitoneal transfer of 2x10^6 IL-10$^+$CD1d^{high} splenic B cells purified from *Schistosoma mansoni*-infected mice successfully prevented ovalbumin-induced airway inflammation through IL-10-dependent induction of pulmonary CD4$^+$CD25$^+$Foxp3$^+$ T cells (Amu et al., 2010). These studies clearly demonstrate the importance of B cells as target cells for helminth parasites. In the present investigation, analysis of the peritoneal cell composition revealed that both macrophages and

B cells account for approximately 30-40% of the whole peritoneal exudate cell population after injection of AvCystatin (data not shown). However, whereas AvCystatin was actively taken up by peritoneal macrophages, $CD19^+$ B cells were not targeted by the immunomodulator. Moreover, intravenous transfer of $2x10^6$ $CD19^+$ B cells from AvCystatin- or control-treated animals into ovalbumin-sensitized recipient mice did not protect against ovalbumin-induced airway hyperreactivity questioning B cells to transfer AvCystatin immunosuppressive effects.

Recent publications highlight a crucial role of dendritic cells in immunomodulation by parasitic worms. *Heligmosomoides polygyrus* infection, for example, was shown to alter phenotypic dendritic cell function in the mucosa thereby protecting mice against colitis (Hang et al., 2010). The depletion of dendritic cells in *Schistosoma mansoni* infection impaired Th2 cytokine production by $CD4^+$ T cells and shifted the host immune response towards IFN-γ production (Phythian-Adams et al., 2010). The present study demonstrated an active endocytotic uptake of AvCystatin by peritoneal macrophages. However, AvCystatin also interacted with $CD11c^+F4/80^-$ cells suggesting that dendritic cells could be altered by AvCystatin. Preliminary data indeed show that treatment with AvCystatin induces a dendritic cell phenotype with intermediate expression of CD80 and CD86 but low expression of MHC-II and CD40 (unpublished data). Such “immature” dendritic cells are ineffective in driving T cell responses and might be important for the induction of tolerance (Lutz and Schuler, 2002). In the present study, co-culture experiments showed that dendritic cells are necessary for the activation of $CD4^+$ T cells since ovalbumin-peptide-treated AvCystatin-MΦ were not sufficent to activate $CD4^+$ T cells (further discussed in **chapter 5.2.5.3**). In order to investigate whether AvCystatin-MΦ modulate dendritic cells to instruct IL-10-producing $CD4^+$ T cells, dendritic cells were pre-cultured with AvCystatin-MΦ in a transwell approach separating the cell populations from each other. Afterwards, dendritic cells were washed and subsequently co-cultured with $CD4^+$ T cells in the absence of macrophages. However, macrophage pre-incubated dendritic cells were not able to induce IL-10 production in $CD4^+$ T cells (data not shown). This shows that dendritic cells are important for $CD4^+$ T cell activation but do not transfer suppressive macrophage effects on $CD4^+$ T cells.

5.2.4 Effects of AvCystatin-MΦ in DSS-induced colitis

To date, millions of people suffer from massive inflammation of the colon accompanied by weight loss, diarrhea, bloody feces, shortening of the colon and mucosal ulceration (Elson et al., 1995; Okayasu et al., 1990). Crohn`s disease (CD) and ulcerative colitis (UC) are considered the two major forms of inflammatory bowel disease (IBD) in humans (Mudter and

Neurath, 2012). Although the etiology has not been fully elucidated, genetic susceptibility, different environmental factors (e.g. smoking or imbalances of the intestinal microbial flora) and immunologic causes (local inflammatory responses of T cells, NK cells or resident macrophages) are speculated to be involved in the development of IBD (reviewed in Hanauer, 2004). To investigate whether AvCystatin-MΦ transfer protection against intestinal inflammation, female C57BL/6 mice were treated with DSS which exerts toxic effects on gut epithelial cells and mimicks human UC in mice (Okayasu et al., 1990). Single transfer of AvCystatin-MΦ prevented body weight loss and shortening of the colon. Histological analysis of distal colon sections further revealed less epithelial/mucosal damage after administration of AvCystatin- but not control-treated macrophages. Moreover, animals treated with AvCystatin-MΦ showed reduced recruitment of neutrophils and eosinophils to the lamina propria. Both cell types are supposed to play a crucial role in the immunopathogenesis of colitis. Eosinophils contribute to tissue destruction through the release of proinflammatory mediators, e.g. major basic protein (MBP), eosinophil cationic protein (ECP) or eosinophil peroxidase (EPO). In a study by Forbes et al (2004), eosinophil-secreted EPO was shown to be critically involved in the development of intestinal inflammation since symptoms of DSS-induced colitis were markedly suppressed in EPO-deficient but not in wildtype mice. Neutrophils accumulate in the colon mucosa during treatment with DSS and secrete cathepsin G, elastase, myeloperoxidase (MPO), lysozyme, lactoferrin and collagenase, factors which have been shown to exert harmful effects on gut tissue (reviewed in Naito et al., 2007). Depletion of neutrophils with specific antibodies or inhibition of neutrophil-secreted products such as elastase was demonstrated to successfully reduce clinical symptoms of colitis in mice (Natsui et al., 1997; Morohoshi et al., 2006).

The amelioration of colitis through transfer of macrophages was reported in a study by Hunter et al. (2010). The investigators stimulated murine peritoneal macrophages *in vitro* with IL-4/IL-13, adoptively transferred these M2a-polarized macrophages intraperitoneally and intravenously into BALB/c mice 48 hours prior to treatment with dinitrobenzene sulfonic acid (DNBS, another chemical shown to induce colitis in mice) and thereby suppressed symptoms of intestinal inflammation. ConA-stimulated splenocytes of mice treated with M2a macrophages produced higher levels of IL-10 compared to control treatment with IFN-γ-stimulated macrophages suggesting a modulatory effect of M2a macrophages on T cells. Neutralization of IL-10 impaired the amelioration of colitis. However, staining of $Foxp3^+$ regulatory T cells determined no significant differences between experimental groups. The investigators thus speculate that the protective effects could also be mediated by IL-10-secreting macrophages or B cells induced by the transferred M2a macrophages. In contrast to Hunter et al. (2010), a study by Brem-Exner et al. (2008) showed that IFN-γ-stimulated

macrophages (IFN-γ-MdC) are profoundly capable of abrogating colitis in mice after tail-vein injection. IFN-γ-MdC deleted intestinal lymphocytes in an *in vitro* co-culture assay and enriched the surviving lymphocytes for $CD4^+CD25^+Foxp3^+$ T cells (Brem-Exner et al., 2008). Compared to the DNBS-colitis model used by Hunter and colleagues, Brem-Exner et al. (2008) induced colitis through transfer of $CD4^+CD62L^+$ T cells into SCID mice. The investigators of the latter study further used bone marrow-derived macrophages instead of peritoneal macrophages for *in vitro* stimulation with IFN-γ. These differences indicate that macrophage functions in murine models of colitis depend on diverse factors such as the mouse strain, the mode of inducing colitis and the cells which are used for macrophage differentiation.

To date, it is not clear whether AvCystatin-MΦ interfere with intestinal inflammation or rather prevent the onset of inflammatory responses by tranquilizing disease-driving cells such as macrophages and thereby suppress granulocyte recruitment. Such a scenario is conceivable since preliminary tracking experiments showed AvCystatin-MΦ not only to migrate to the mesenteric lymph nodes and to the colon but also to locally proliferate in these compartments (data not shown). Adoptive transfer of AvCystatin-MΦ one day after access to DSS did not induce Foxp3-expressing regulatory T cells (data not shown). Moreover, ConA-stimulation of splenocytes, lamina propria cells or mesenteric lymph node cells of mice treated with AvCystatin-MΦ did not induce an IL-10 response by the respective cells (data not shown). This is not surprising as DSS-induced colitis reflects the pathology of acute colitis which is driven by macrophages and granulocytes rather than by $CD4^+$ T cells. Strikingly, these findings show that AvCystatin-MΦ in DSS-induced colitis act via a mechanism which is different from the mechanism determined for ovalbumin-induced airway hyperreactivity. The inhibition of colonic epithelial cell-derived chemokines by AvCystatin-MΦ might constitute an alternative mode of suppression. IL-8, for example, represents an important neutrophil chemoattractant which is increased in the mucosa of human patients with UC (Isaacs et al., 1992; reviewed in Naito et al., 2007). In the murine system no IL-8 homologue has been identified but macrophage inflammatory protein 2 (MIP-2), keratinocyte chemoattractant (KC or CXCL1) or lipopolysaccharide-induced CXC chemokine (LIX) are important neutrophil chemoattractants in mice (Rovai et al., 1998; Song et al., 1999). Eotaxins produced by macrophages or intestinal epithelial cells are essential for eosinophil recruitment to the colon (Ahrens et al., 2008). By analysing colon homogenates in the present study, no significant differences in eotaxin expression between experimental groups were detected (data not shown). Another mechanism by which AvCystatin-MΦ might execute effects on the development of colitis is to influence tight or gap junction functions. Cytokines such as IFN-γ, HGF, TNF-α, EGF and TGF-β1 have been described to modulate the

structure and function of tight junctions *in vitro* and *in vivo* thereby affecting paracellular permeability (reviewed in Walsh et al., 2000). Moreover, LPS/IFN-γ-activated macrophages were shown to inhibit enterocyte gap junctions by suppressing the phosphorylation of connexin 43 (Cx43) in an NO-dependent manner. NO production by the M1-polarized macrophages led to an internalization of Cx43 in adjacent enterocytes and efficiently inhibited enterocyte migration (Anand et al., 2008). Thus, it is possible that AvCystatin-MΦ either directly secrete specific mediators which regulate barrier functions in the colon or, indirectly, influence intestinal epithelial cells to produce such factors.

5.2.5 Macrophage profiling

To gain insights into the macrophage-mediated immunosuppression, transcriptional profiling of AvCystatin-MΦ was performed. *In vitro* and *in vivo* stimulation with AvCystatin initially up-regulated pro- and anti-inflammatory marker genes such as iNOS, IL-12/23p40, TNFα, IL-6 and IL-10 in peritoneal macrophages. However, at a later timepoint, the macrophage signature changed to an M2a/M2b hybrid phenotype which was characterized through the expression of SPHK-1, LIGHT and ARG-1.

5.2.5.1 Phenotypic plasticity of AvCystatin-MΦ

It has been reported that nematode-secreted molecules often generate pro-inflammatory activity before gradually changing to an anti-inflammatory response (Goodridge et al., 2005 a). Since macrophages show high phenotypic and functional plasticity, the initial expression of pro-inflammatory markers by AvCystatin-MΦ before switching to a suppressive phenotype is not unusual. Although it is not completely clear whether phenotypical changes are a result of cell differentiation into another activation state or whether new macrophage populations are recruited to the tissue and replace the original cells (Mosser and Edwards, 2008), a rising number of publications substantiate that macrophages adapt their phenotypic properties to environmental changes. Phenotypical conversion was reported for macrophages in cancer which shift from classical activation to an alternative regulatory status and for macrophages in obesity that switch from wound-healing activity to classical macrophage activation (reviewed in Mosser and Edwards, 2008). In a study using the C2D macrophage cell line, Potts et al. (2008) found that macrophages respond to *in vivo* signals that are absent *in vitro*. After intraperitoneal injection of untreated C2D macrophages, cells differentiated from an immature phenotype to a mature activation stage which expressed increased levels of c-fms, F4/80 and CD11b thereby substantiating that the *in vivo* environment affects phenotypic characteristics of macrophages. Treatment of M2b macrophages with serum amyloid P component (SAP), an acute-phase protein in mice, switched M2b-polarized macrophages

towards an M2a phenotype through the activation of PI3K/AKT-ERK (Zhang et al., 2011). Similarly, PPARγ ligands were shown to induce a phenotypic conversion of macrophages from M2b towards M2a activation thereby promoting mucosal defense against gastrointestinal candidiasis (Lefèvre et al., 2010). Stout et al. (2005) found that changes of microenvironmental conditions affect and alter the functional phenotype of macrophages. Moreover, Mylonas et al. (2009) reported on helminth-derived macrophages that could be reprogrammed to perform microbial killing. In the present investigation *in vivo*- and *in vitro*-generated AvCystatin-MΦ showed similar gene expression. Since the recruitment of additional macrophage populations is not possible *in vitro*, AvCystatin-MΦ seem to shift their phenotype from an early M1-like to a suppressive profile. This excludes the possibility that the determined phenotype is an agglomeration of different macrophage subpopulations that have been recruited to the peritoneum after treatment with AvCystatin. A stable expression of SPHK-1, LIGHT and ARG-1 for at least seven days was determined for *in vitro*-stimulated AvCystatin-MΦ (data not shown). However, whether AvCystatin-MΦ maintain their transcriptional signature after being transferred into disease models or rather adapt their phenotype to the prevailing microenvironmental conditions is not yet known.

The expression of “early” and “late” marker genes in AvCystatin-MΦ was identical (except for the expression of IL-10) in macrophages from wildtype and IL-10-deficient mice suggesting that the phenotypic conversion of AvCystatin-MΦ is IL-10 independent. By characterizing gene expression in LPS-tolerogenic macrophages, Porta et al., (2009) found NF-kappaB subunit p50 to limit the recruitment of polymerase II on M1 promoter genes which led to a suppression of STAT1 activation, the inhibition of IFN-β production and M1-polarization of murine peritoneal macrophages. Contrariwise, NF-kappaB p50 supported the recruitment of polymerase II on M2 promoter genes thereby driving M2 macrophage polarization through up-regulation of M2-related genes such as IL-10, TGF-β, ARG-1, CCL2, CCL17 and CCL22. Thus, NF-kappaB p50 may constitute a potent negative regulator of M1 gene expression but an important inducer of M2 macrophage polarization (Porta et al., 2009). Preliminary experiments show that NF-kappaB p50 activity might also be important for phenotypic changes in AvCystatin-MΦ. As reported earlier, AvCystatin induced expression of SPHK-1, LIGHT and ARG-1 in peritoneal macrophages. The induction of these markers, however, was reduced in macrophages of p50-deficient mice (data not shown). In summary, phenotypical changes of macrophages are an important and defining feature of macrophage plasticity. Such flexibility also applies for AvCystatin-MΦ which convert their phenotype from M1 to M2a/M2b activation independent of IL-10 but possibly through the activity of the NF-kappaB subunit p50.

5.2.5.2 AvCystatin-MΦ hybrid phenotype

The identification and characterization of macrophage subpopulations is highly relevant to the understanding of disease pathogenesis but also for diagnostic or therapeutic intervention. The currently used concept of murine macrophage classification distinguishes M1 and M2 (M2a, M2b and M2c) polarization. Since macrophage activation is not static, this classification might be oversimplified. Three main aspects have been proposed to influence the diversity of macrophage activation. First the complexity of the environmental milieu, second the sequence of exposure to the stimuli and finally the status of macrophage differentiation at the time of its exposure (Stempin et al., 2009). Therefore, Mosser and Edwards (2008) proposed a classification system that reflects macrophage diversity more adequately by including macrophages with overlapping expression profiles and functions. The respective authors also suggested the existence of hybrid macrophage populations by stimulating murine macrophages with a combination of IL-4 and LPS in the presence of immune complexes. The resulting macrophage population expressed RELM-α and showed hypersecretion of IL-10 thus sharing characteristics of M2a and M2b macrophages (Mosser and Edwards, 2008). In the present study, AvCystatin-MΦ were found to show characteristics of M2b macrophages by expressing SPHK-1, LIGHT and features of M2a activation by expressing ARG-1 further supporting the idea of macrophage subsets with overlapping phenotypes.

5.2.5.3 Biological functions of specific AvCystatin-MΦ markers

SPHK-1 is a lipid kinase which phosphorylates sphingosine to sphingosine-1-phosphate (S1P), a lysosphingophospholipid, which either acts as a second messenger within cells or extracellularly binds to a specific group of G-protein-coupled receptors (S1PR1-S1PR5). In mammals, S1P predominantly occurs in the blood or lymph (Hla et al., 2008) and, depending on which cell type and receptor is addressed, exerts diverse biological functions. S1P was shown to suppress $CD4^+$ T cell proliferation (Jin et al., 2003), to promote cell migration and proliferation and to regulate calcium mobilization and apoptosis (Spiegel and Milstien, 2003). In a study by Wang et al. (2005), S1P was found to mediate immunosuppressive effects on T cells through the S1P-S1PR4 axis. Stimulation of the mouse $CD4^+$ T cell lines D10G4.1 and EL4.IL-2 with S1P in a dose-depedent manner after activation with anti-CD3/anti-CD28 suppressed $CD4^+$ T cell proliferation, inhibited the production of IL-2, IL-4 and IFN-γ but increased the secretion of IL-10. These findings closely reflect the effects of AvCystatin-MΦ on ovalbumin-specific $CD4^+$ T cells. Moreover, it has been shown that SPHK-1 is activated by ERK-mediated phosphorylation at position Ser225 (Pitson et al., 2003). It is therefore conceivable that AvCystatin-induced phosphorylation of ERK in macrophages is important for

the activation of SPHK-1. However, by using a S1PR antagonist and supplementing cultures of dendritic cells and $CD4^+$ T cells with S1P, no evidence was found for a S1P-mediated induction of $CD4^+IL\text{-}10^+$ T cells. S1P was also elucidated to exert functions on macrophage biology. Thus, it is possible that S1P acts back on AvCystatin-MΦ and thereby equips the suppressive macrophage population with additional functions. Different studies show that S1P is critical for macrophage trafficking (Gude et al., 2008), protects macrophages against apoptosis (Weigert et al., 2007), mediates phagosome maturation by modulating actin assembly (Anes et al., 2003) and drives anti-inflammatory activation of macrophages (Hughes et al., 2008). In a recent investigation, S1P further increased antibody-mediated phagocytic activity of alveolar macrophages through the interaction with S1PR2 and regulation of Fcγ receptor expression (McQuiston et al., 2011). These findings favour the idea that S1P could also influence Fcγ receptor expression in AvCystatin-MΦ. Surface marker analysis revealed an up-regulation of CD16/32 on AvCystatin-MΦ which was reflected by real-time PCR analysis of CD32 (Fcγ receptor II) RNA expression. RNA levels of CD64 (Fcγ receptor I) and CD16 (Fcγ receptor III), however, were decreased (data not shown). Murine macrophages express two S1P receptors, namely S1PR1 and S1PR2 (Duong et al., 2004; Hughes et al., 2008). In order to analyse effects of S1P on AvCystatin-MΦ functions, S1P receptors on macrophages could be blocked by the specific antagonists VPC23019 (S1PR1) and JTE013 (S1PR2) and the macrophages evaluated for suppressive activity or additional functions such as trafficking properties or protection against apoptosis.

LIGHT is a member of the tumor necrosis family and has also been desinated as TNF superfamily member 14 (TNFSF14) or CD258. It is expressed by activated immune cells such as T cells, macrophages, monocytes, granulocytes and dendritic cells (Mauri et al., 1998; Tamada et al., 2000) and constitutes a 29 kDa type II homotrimer transmembrane protein. However, LIGHT can also be cleaved from the cell surface (Mauri et al., 1998, Edwards et al., 2006). It binds two receptors, the herpesvirus entry mediator (HVEM) and the lymphotoxin β receptor (LTβR) and was shown to transduce co-stimulatory signals into $CD4^+$ T cells through HVEM (Wan et al., 2002; Shi et al., 2002; Tamada et al., 2000). Since the interaction between LIGHT and T cells has been reported, soluble LIGHT (sLIGHT) theoretically represents a candidate for the induction of IL-10 in $CD4^+$ T cells after treatment with AvCystatin-MΦ. On the other side, there are no studies which provide evidence for an IL-10-inducing function of LIGHT. Moreover, M2b macrophages which express and secrete LIGHT (Edwards et al., 2006) did not induce $CD4^+IL\text{-}10^+$ T cells in the established co-culture assay. Therefore, it is questionable whether AvCystatin-MΦ-derived LIGHT mediates IL-10 induction in $CD4^+$ T cells. LIGHT has been addressed as a candidate for cancer treatment as it up-regulated ICAM-1 which induced tumor cell apoptosis (Zhai et al., 1998; Rooney et al.,

2000). AvCystatin-MΦ were found to up-regulate ICAM-1. Hence, it will be interesting to address whether (a) the expression of LIGHT in AvCystatin-MΦ is required to induce ICAM-1 and (b) ICAM-1 on AvCystatin-MΦ can induce apoptosis in tumor cells. Doherty et al. (2011) showed that LIGHT is important for airway remodelling in mouse models of chronic asthma. Inhibition of LIGHT in the respective study impaired fibrosis, smooth muscle accumulation and suppressed the expression of lung TGF-β. This hints towards a possible function of AvCystatin-MΦ-derived LIGHT for lung regenerative functions during airway inflammation. Tracking of AvCystatin-MΦ showed that the macrophages reside in the spleen and PBLNs. By reason that AvCystatin-MΦ were not directly tracked in the lungs, it is likely that they mediate suppression on lung inflammation by inducing IL-10-producing lymphocytes rather than mediating local regenerative effects on lung tissue as described by Doherty et al. (2011). By investigating the influence on macrophage functions, LIGHT was found to act as a mediator for macrophage migration associated with the activation of signalling kinases such as MAPK, PI3K/AKT, NF-kappaB, Src members, and FAK (Wei et al., 2006). MAPK p38 and ERK were phosphorylated in macrophages after treatment with AvCystatin. Preliminary data further indicate that induction of LIGHT in AvCystatin-MΦ is associated with the NF-kappaB subunit p50. Whether expression of LIGHT in AvCystatin-MΦ is correlated with macrophage migration as shown in Wei et al. (2006) is not yet known.

ARG-1 is a typical M2a activation marker of murine macrophages and commonly expressed after exposure to IL-4 and IL-13. ARG-1 is localized in the cytoplasm of liver cells but can also be expressed in other cell types after appropriate stimuli (Morris, 2000). In mice, ARG-1 is mainly induced in macrophages. In the human system, it is constitutively expressed in granulocytes (reviewed in Stempin et al., 2009). ARG-1 has been shown to act in a context-dependent manner. It either protects the host against parasite (re)infection or negatively regulates Th2 responses by suppressing T cell activity through L-arginine depletion that leads to CD3 instability (Modolell et al., 2009). AvCystatin-MΦ modulated $CD4^+$ T cell responses suggesting that ARG-1 could be involved in this effect. Inhibition of ARG-1 through the inhibitor BEC, however, did not reverse this effect indicating that T cell modulation by AvCystatin-MΦ is ARG-1-independent. Another feature of ARG-1 is the ability to mediate tissue recovery through production of polyamines and collagen. *Acanthocheilonema viteae* is a nematode that migrates through the host`s body and thereby causes tissue injury. The induction of ARG-1-expressing macrophages by AvCystatin seems to be beneficial for the parasite to mediate tissue remodelling and prevent inflammatory host responses induced through tissue damage. Whereas helminth-induced ARG-1 expression in macrophages mainly depends on IL-4/IL-13-mediated activation of STAT6, intracellular pathogens such as *Mycobacterium tuberculosis* or *Toxoplasma gondii* can induce ARG-1

independent of STAT6 activation via the TLR pathway (El Kasmi et al., 2008). AvCystatin was actively taken up by peritoneal macrophages and *in vivo* as well as *in vitro* induced a specific macrophage phenotype. The absence of Th2 cells in the *in vitro* macrophage culture (purity ≥ 95%) denotes that the induction of ARG-1 in AvCystatin-MΦ does not depend on Th2 cytokines but is rather directly mediated by the immunomodulator. AvCystatin induces MAPK-mediated activation of STAT-3 and CREB in macrophages (Klotz et al., 2011 b). Whether AvCystatin in addition addresses the STAT-6 pathway or an alternative pathway to induce ARG-1 in macrophages is unclear.

Besides transcriptional analysis, the *in vivo* effect of AvCystatin on macrophage surface marker expression was determined by flow cytometry analysis. AvCystatin induced up-regulation of MHC-II, costimulatory molecules (CD40, CD80, CD86), ICAM-1, Fcγ receptors (CD16/32), PD-L1 and PD-L2 18-20 hours after intraperitoneal application into mice. Whereas M1-polarized macrophages are known to exhibit enhanced antigen-presenting and anti-microbial activity, M2 macrophages are described to be less efficient APCs. Schebesch et al. (1997) however, showed that this does not necessarily depend on decreased expression of MHC-II or costimulatory markers since CD11a, CD40, CD54, CD58, CD80 and CD86 were similarly expressed in M1 and M2 macrophages. Thus, M2 macrophages in principle exhibit all requirements to drive immune responses (Noel et al., 2004). IL-10-hypersecreting M2b macrophages, for example, express high amounts of MHC-II, CD86 and beyond that can efficiently activate $CD4^+$ T cells through antigen-presentation (Edwards et al., 2006). AvCystatin-MΦ show similarities with M2b macrophages. However, in the absence of dendritic cells they are insufficient to drive T cell responses. The inability of AvCystatin-MΦ to activate $CD4^+$ T cells was observed when ovalbumin-peptide-treated AvCystatin-MΦ were *in vitro* incubated with ovalbumin-specific $CD4^+$ T cells and failed to induce antigen-specific cytokine responses in $CD4^+$ T cells (data not shown). It is likely that this discrepancy between M2b macrophages and AvCystatin-MΦ results from metabolic differences. After being activated, murine macrophages metabolize the amino acid L-arginine in two competitive pathways. In the presence of Th1 stimuli, macrophages convert L-arginine into NO through iNOS. This pathway represents a key element to control and eliminate intracellular pathogens by increased cytotoxic and antimicrobial activity. Under Th2 conditions, ARG-1 is expressed which catalyzes the conversion of L-arginine into proline, L-orthinine and polyamines which are important factors for cell growth, collagen formation, and tissue repair (Hesse et al., 2001). This alternative pathway induces macrophages which exhibit regulatory and recovery functions rather than effector functions that lead to pathogen killing (Mosser, 2003). The competitive nature of the two metabolic pathways was nicely reflected by the opposed expression of iNOS and ARG-1 in AvCystatin-MΦ. Whereas treatment with

AvCystatin led to an early expression of iNOS in macrophages, ARG-1 was induced after the observed conversion to a suppressive phenotype. M2b macrophages are described to exhibit similarities with M1 macrophages since they also produce low amounts of pro-inflammatory marker genes, e.g. iNOS and do not exert ARG-1 activity (Mosser, 2003). AvCystatin-MΦ, in contrast, did not show expression of pro-inflammatory markers 18-20 hours after treatment with AvCystatin but expressed ARG-1 in addition to the M2b activation markers SPHK-1 and LIGHT. Hence, although M2b and AvCystatin-MΦ share characteristics, M2b macrophages might be more orientated towards the classical metabolic pathway whereas AvCystatin-MΦ show higer affinity to the alternative pathway. This could explain why AvCystatin-MΦ in comparison to M2 macrophages fail to activate T cells.

Similar to the expression of ICAM-1 in AvCystatin-MΦ, murine alveolar macrophages were shown to up-regulate ICAM-1 during infection with *Nippostrongylus brasiliensis* (Siracusa et al., 2008). The functional role of ICAM-1 in helminth-derived or AvCystatin-induced macrophages remains speculative. Studies on ICAM-1 functions in rats and mice showed that expression of ICAM-1 on alveolar epithelial cells increases the adhesiveness of alveolar macrophages (Ward, 2003). ICAM-1 on alveolar macrophages was found to be associated with the actin cytoskeleton (Barton et al., 1996) thereby promoting macrophage mobility in the alveolus and in addition to facilitate phagocytosis of particulates by alveolar macrophages (Paine et al., 2002). ICAM-1 binds to integrins on endothelial cells and mediates transmigration of leucocytes or macrophages into tissues. In context of the present study, this could suggest that ICAM-1 supports the migration of AvCystatin-MΦ into lung tissue. Since AvCystatin-MΦ were not detected in the lung, it is possible that they initially migrate to the lung, interact with local cells (e.g. epithelial cells) before migrating to secondary lymphoid organs such as the spleen or the PBLNs. To determine a precise migration pattern of AvCystatin-MΦ, continuative *in vivo* tracking is necessary which includes the analysis of additional timepoints and tissues. As mentioned above, ICAM-1 was shown to induce apoptosis in tumor cells (Zhai et al., 1998; Rooney et al., 2000). Thus, it is feasible that AvCystatin-MΦ could also exert tumoricidal effects through ICAM-1.

After treatment with AvCystatin, macrophages up-regulated Fcγ receptors which constitute a family of receptors that is important for the recognition of IgG Fc fragments. Interestingly, crosslinking of Fcγ receptors by immune complexes in the presence of TLR ligands induce IL-10-hypersecreting type 2 (M2b) macrophages. To date, one inhibitory Fcγ receptor (Fcγ receptor II) and three activating Fcγ receptors (Fcγ receptor I, III and IV) are described for mice. Whereas Fcγ receptor I (CD64) is permanently saturated with monomeric IgG, Fcγ receptor II (CD32) and Fcγ receptor III (CD16) show low affinity to monomeric IgG but

instead are efficient in binding IgG-IC (Nimmerjahn and Ravetch, 2006). As discussed earlier, it is possible that AvCystatin addresses Fcγ receptors to modulate macrophage functions. However, in that case AvCystatin would have had to directly bind Fcγ receptors as the *in vivo* generation of AvCystatin immune complexes (AvCystatin-IC) within a time frame of 18-20 hours is rather unlikely. Apart from that, initial *in vitro* experiments showed that treatment of macrophages with AvCystatin-IC in combination with LPS induced lower expression of iNOS and IL-12/23p40 but increased transcript levels of IL-10 and SPHK-1 whereas LIGHT and ARG-1 expression was almost equal when compared to AvCystatin treatment (data not shown). These preliminary findings suggest that using AvCystatin-IC instead of AvCystatin could be a powerful tool to induce a macrophage population with enhanced immunosuppressive properties.

PD-L2 is commonly expressed on M2 macrophages and negatively regulates $CD4^+$ T cell activation, suppresses T cell proliferation and dampens Th2-allergic inflammation (Zhang et al., 2006; Huber et al., 2010). *Nippostrongylus brasiliensis* was determined to induce PD-L2 on murine macrophages in an IL-4/IL-13 and STAT6-dependent manner. *In vivo* blockade of PD-L2 in the respective study increased Th2 responses in the lung of infected mice suggesting a Th2-silencing function of PD-L2 (Huber et al., 2010). In a murine model of asthma, PD-L2-deficient mice showed exacerbated airway inflammation whereas opposing effects were demonstrated for PD-L1-deficient mice (Akbari et al., 2010). PD-L1 expression has originally been attributed to classical macrophage activation. However, studies using different murine helminth infection models show that PD-L1 can also be up-regulated on M2 macrophages and successfully inhibit $CD4^+$ T cell responses. Blockade of PD-L1 and PD-L2 on *Taenia crassiceps*-induced macrophages, for example, reversed $CD4^+$ T cell proliferation in response to parasite antigens (Terrazas et al., 2005). Moreover, in a murine model of *Schistosoma mansoni* infection, PD-L1 was selectively up-regulated on splenic macrophages and shown to induce $CD4^+$ T cell anergy (Smith et al., 2004). A new function of PD-L1 was demonstrated by its ability to promote the induction and maintenance of Foxp3-expressing regulatory $CD4^+$ T cells (Francisco et al., 2009). The investigators generated $Foxp3^+$ regulatory T cells *in vitro* by using PD-L1-coated beads and found PD-L1 to induce conversion of naive $CD4^+$ T cells into regulatory $CD4^+$ T cells through inhibition of phospho-AKT, mammalian target of rapamycin (mTOR), S6 ribosomal protein, ERK2 but up-regulation of the phosphatase and tensin homolog (PTEN). PD-L1 further maintained the expression of Foxp3 on the induced regulatory T cells. Thus, both PD-L1 and PD-L2 are important molecules for cell contact-dependent inhibition of $CD4^+$ T cell functions. AvCystatin-MΦ showed up-regulation of both molecules 18-20 hours after treatment with the immunomodulator. The induction of $CD4^+IL\text{-}10^+$ T cells, however, was determined to be cell

contact-independent. In order to definately exlude a role of programmed death ligands or a possible additive effect on the IL-10 induction in $CD4^+$ T cells, both molecules were blocked by using anti-PD-L1 and anti-PD-L2 antibodies described in Terrazas et al. (2005). Blockade of PD-L1 and PD-L2, however, had no effects on the AvCystatin-MΦ-mediated IL-10 induction in $CD4^+$ T cells.

In summary, treatment with AvCystatin induced the expression of macrophage markers which exert important immunoregulatory functions and thus constitute important candidates for the AvCystatin-MΦ-mediated immunosuppression.

5.2.6 Mechanistical analyses of IL-10 induction by AvCystatin-MΦ

5.2.6.1 IL-10 induction in $CD4^+$ T cells by different macrophage populations

Transcriptional profiling of AvCystatin-MΦ determined similarities to M1 macrophages initially after stimulation and characteristics of M2a and M2b macrophage activation at a later timepoint. Specific properties of AvCystatin-MΦ with respect to the induction of IL-10 in T cells were determined by *in vitro* experiments incubating M1, M2a and M2b macrophages with ovalbumin-peptide-treated dendritic cells and ovalbumin-specific $CD4^+$ T cells. M1 and M2a macrophages showed no self-production of IL-10 after stimulation with the respective stimuli whereas higher levels of IL-10 were secreted by M2b macrophages. This is in line with reports that describe M2b macrophages as IL-10-hypersecreting cells. IL-10 production by M2a macrophages was reported in a study by Zhang et al. (2011). Other studies, however, describe M2a macrophages as an inefficient source for IL-10 (Edwards et al., 2006). In the present investigation, a significant increase of IL-10 was only detected in culture supernatants of dendritic cells and $CD4^+$ T cells in the presence of *in vivo*-generated AvCystatin-MΦ but not after incubation with M1, M2a or M2b macrophages. Since *in vitro*-treated macrophages were less effective inducers of IL-10 production in $CD4^+$ T cells, it can be assumed that specific environmental conditions within the peritoneal cavity are necessary to equip AvCystatin-MΦ with IL-10-inducing properties. Analysis of the cell composition in the peritoneal cavity of AvCystatin-treated mice revealed an influx of $Ly6C^+$ monocytes which was not detectable in control-treated animals. These cells, however, were completely depleted through biotinylated anti-Gr1 antibodies during macrophage purification and thus do not directly contribute to the protective macrophage effects in ovalbumin-induced airway hyperreactivity (data not shown). However, it is possible that recruited monocytes interact with resident peritoneal macrophages during a short time frame and thereby mediate IL-10-inducing properties to these cells. Together, these findings show that AvCystatin-MΦ share

similarities with M1, M2a and M2b macrophages on a transcriptional basis but, in addition, exhibit regulatory properties which are distinct from these macrophage populations.

5.2.6.2 Evaluation of specific markers for the IL-10 induction in $CD4^+$ T cells

SPHK-1, ARG-1 and programmed death-ligands have been described to exert regulatory effects on $CD4^+$ T cells (Wang et al., 2005; Pesce et al., 2009 b; Francisco et al., 2009). SPHK-1 phosphorylates sphingosine to S1P which binds to S1P receptors (S1PR) and thereby can affect $CD4^+$ T cell responses. In order to prevent binding of S1P to S1PR1, ovalbumin-specific $CD4^+$ T cells were pre-treated with different concentrations of the S1PR1 antagonist VPC23019 and thereafter incubated with ovalbumin-peptide-treated dendritic cells and macrophages. The efficiency of VPC23019 antagonism has formerly been described by Osada et al. (2002) and Davis et al. (2005). Blockade of S1PR1 by VPC23019 did not influence the AvCystatin-MΦ-induced IL-10 production by ovalbumin-specific $CD4^+$ T cells in the established co-culture system. However, this finding has to be handled with care. Besides S1PR1, another S1PR, namely S1PR4, is expressed on murine $CD4^+$ T cells. To date, no antagonists are available for S1PR4. Thus, despite blocking the S1P-S1PR1 axis through VPC23019, S1P could still have induced IL-10 through S1PR4. In order to prove this possibilitiy, $CD4^+$ T cell and dendritic cell co-cultures were supplemented with S1P in a dose-dependent manner. Supplementation with S1P, however, did not induce IL-10 secretion by $CD4^+$ T cells. The induction of IL-10 through S1P has been demonstrated in a publication by Wang et al. (2005). The authors showed that the mouse Th2 T cell line D10G4.1 secreted increased amounts of IL-10 after activation with anti-CD3/anti-CD28 and treatment with S1P via the S1P-S1PR4 axis. In the present investigation, S1P-mediated IL-10 induction could not be verified in ovalbumin-specific $CD4^+$ T cells of DO11.10 mice suggesting that this could be a specific effect of D10G4.1 cells. In order to study the functions of S1PR4 in T cells, Wang and colleagues experimentally modified D10G4.1 cells through the transfection of an insert encoding for the mouse S1PR4 gene and further treated the cells with FTY720, a derivative of myriocin (ISP-1) from the fungus *Iscaria sinclarii*, to down-regulate S1PR1 prior to treatment with S1P (Wang et al., 2005). The results of the present study, however, hint towards an irrelevant function of SPHK-1/S1P for the observed IL-10 induction in $CD4^+$ T cells. This is substantiated by the finding that M2b macrophages, which have been well characterized for high expression of SPHK-1 (Anderson and Mosser, 2002; Edwards et al., 2006), did not induce IL-10 in $CD4^+$ T cells in the established *in vitro* co-culture system. Further experiments either using the ARG-1 inhibitor BEC or programmed death ligand neutralizing antibodies anti-PD-L1 and anti-PD-L2 did not reveal a suppression of IL-10 levels in co-culture supernatants. The functionality of the ARG-1 inhibitor was tested by

measuring urea in the supernatant of *in vitro*-generated M2a macrophages either cultured alone or in the presence of different BEC concentrations. Moreover, both anti-PD-L1 and anti-PD-L2 antibodies have formerly been demonstrated to efficiently inhibit effects of macrophage-expressed PD-L1 and PD-L2 on $CD4^+$ T cell responses (Terrazas et al., 2005). Collectively, these findings exclude the induction of IL-10-producing $CD4^+$ T cells by the AvCystatin-MΦ markers SPHK-1, ARG-1, PDL-1 and PD-L2. These markers might be descriptive for the macrophage phenotype or important for other macrophage functions but dispensible for the induction of $CD4^+IL\text{-}10^+$ T cells.

5.2.6.3 Factors which are described to induce IL-10 in T cells

A couple of soluble factors have been described as inducers of IL-10 in $CD4^+$ T cells. Pro-inflammatory cytokines, for example, induce IL-10 to regulate immune responses and to prevent overshooting reactions (reviewed in Couper et al., 2008). IL-12 in combination with high antigen doses was reported to induce IL-10-producing $CD4^+$ Th1 T cells via phosphorylation of ERK and activation of STAT4 (Saraiva et al., 2009). IL-27 has been shown to differentiate naïve $CD4^+$ T cells into IL-10-producing cells and, in combination with TGF-β, enhance Tr1 development (Awasthi et al., 2007). Stumhofer et al. (2007) demonstrated that IL-27 and IL-6 can independently induce IL-10-producing $CD4^+$ T cells under different microenvironmental conditions. This effect was dependent on STAT1 and STAT3 activation for IL-27 and STAT3 signalling for IL-6. NO induced the development of $CD4^+CD25^+Foxp3^-$ regulatory T cells (NO-Tregs) which attenuated colitis and collagen-induced arthritis in an IL-10-dependent manner after intraperitoneal injection into mice (Niedbala et al., 2007). In the present investigation, macrophage expression of IL-12p40, IL-6 and iNOS peaked between 1 and 5 hours after contact with AvCystatin and declined afterwards. Successional experiments verified a similar expression of IL-27 in AvCystatin-MΦ (data not shown). For *in vitro* incubation with dendritic cells and $CD4^+$ T cells, macrophages were isolated from mice 18-20 hours after intraperitoneal administration of AvCystatin. To this timepoint, AvCystatin-MΦ had already changed from M1 activation to a suppressive M2a/M2b hybrid phenotype which did not express pro-inflammatory gene transcripts or secrete the respective cytokines. Moreover, no IL-10 induction in T cells was determined through the secretion of pro-inflammatory cytokines by M1 macrophages in the established co-culture assay. Together, these findings challenge IL-12, IL-6, NO and IL-27 to be mediators for the observed IL-10 induction in ovalbumin-specific $CD4^+$ T cells. Nevertheless, it will be useful to block receptors on and pathways in $CD4^+$ T cells addressed by these cytokines and to evaluate IL-10 production by T cells in co-culture assays with AvCystatin-MΦ and dendritic cells. In a study by Spolski and co-workers (2009), IL-21 was determined

as an important inducer of IL-10 in naïve $CD4^+$ T cells, Th1 $CD4^+$ T cells or Tc1 T cells which constitute a subpopulation of $CD8^+$ cytotoxic T cells (Spolski et al., 2009). IL-21 is produced by different subpopulations of $CD4^+$ T cells and by NK T cells (Spolski et al., 2008) questioning macrophages as a cellular source for this cytokine. One can speculate that AvCystatin-MΦ induce IL-21 secretion by activated $CD4^+$ T cells which finally acts back on T cells thereby inducing IL-10 production. This scenario, however, is rather unlikely since $CD4^+$ T cells produced IL-10 already 48 hours after contact with AvCystatin-MΦ. Such early production of IL-10 by naïve T cells is surprising and presumably not induced by an autocrine IL-21 feedback mechanism. Induction of IL-10 in IFN-γ-secreting $CD4^+$ Th1 cells has been determined for notch ligands of the delta-like family (DII-1 and DII-4) in combination with costimulation by IL-12 or IL-27 in a STAT4-dependent manner (Rutz et al., 2008). Expression of notch ligands by macrophages was reported for the treatment of macrophages with schistosomal egg antigen (Goh et al., 2008) and during influenza H1N1 virus infection (Ito et al., 2011). Whether notch ligands are induced in AvCystatin-MΦ has to be determined. At present, the secretion of notch ligands by AvCystatin-MΦ is theoretically possible and thus represents a feasible mechanism for the induction of IL-10 in $CD4^+$ T cells. Preliminary data further indicate that Fas ligand (FasL), a type-II transmembrane protein of the tumor necrosis factor family, is up-regulated in AvCystatin-MΦ. Yang et al. (2003 a) showed that the inhibition of Fas/Fas ligand interaction by antagonistic antibodies prevents IL-10 induction in human Jurcat cells, an immortalized T lymphocyte cell line that is preliminary used to study issues in cancer and T cell signalling. In another study, soluble FasL (sFasL) has been reported to modulate IL-10 transcription and secretion in human $CD8^+$ lymphocytes and neutrophils since depletion of sFasL suppressed IL-10 modulation in the respective cells (Giho et al., 2010). Thus, Fas ligand constitutes another possible candidate for the induction of $CD4^+IL\text{-}10^+$ T cells by AvCystatin-MΦ. Additional investigations showed that AvCystatin-MΦ-secreted components only induced IL-10-producing T cells if they were freshly added to activated $CD4^+$ T cells. Frozen and thawed macrophage supernatants, however, failed to induce IL-10 in $CD4^+$ T cells (data not shown). Moreover, supernatants from AvCystatin-MΦ cultured in FCS-free medium were not capable in inducing $CD4^+IL\text{-}10^+$ T cells (data not shown). These results hint towards a mediator that is rather unstable and probably gets stabilized through FCS after being secreted by the macrophages. Thus, it is important to identify single candidates by the analysis of AvCystatin-MΦ supernatants and to evaluate their potential to instruct IL-10-producing $CD4^+$ T cells.

5.2.7 Clinical impact of regulatory macrophages

At present, glucocorticoids and antihistamines are most commonly used to treat allergy-related disorders. The disadvantages are side effects like osteoporosis, the need to take drugs life-long and an unsatisfactory disease control in 30% of allergic patients (Hawrylowicz and O`Garra, 2005). Autoimmune diseases are commonly treated with immunosuppressant drugs, e.g. corticosteroids which, in the long run, can lead to diabetes, eye disease (glaucoma or cataract), stomach ulcers, high blood pressure and depression. Treatment with living parasites to allay or cure inflammatory bowel disease in human patients has successfully been demonstrated (Summers et al. 2005; Croese et al., 2006). However, although the concept of worm therapy has been described as safe and effective, the application of living parasites always bear the risk of unwanted side effects. Therapeutic intervention by applying helminth-secreted immunomodulators is another strategy to treat inflammatory responses. Such products, however, often exert immunogenic activity. Thus it might be critical to use helminth-derived molecules as pharmaceuticals. Cell-based therapies constitute a novel approach in which cells are differentiated into a specific activation stage and finally administered into patients in order to transfer immunosuppressive properties. Macrophages represent good candidates for cell-based therapeutic intervention not only for atopic and autoimmune diseases but also for the treatment of kidney diseases or to promote tolerance to transplanted organs (**Table 5-1**).

A case report by Hutchinson and colleagues described the successful treatment of a human kidney transplant recipient with central venous infusion of IFN-γ-treated transplant acceptance-inducing cells (TAICs) which show similarities to unactivated, tissue-resident macrophages and possess $CD4^+$ T cell suppressive functions (Hutchinson et al., 2008 and 2009). The mouse equivalent of human TAICs is a macrophage derivative which has been designated as IFN-γ-stimulated monocyte-derived cells (IFN-γ-MdCs). IFN-γ-MdCs were differentiated from bone marrow-derived macrophages (BMMΦ) through IFN-γ treatment. Intravenous transfer of $5x10^6$ IFN-γ-MdCs into SCID mice successfully mitigated $CD4^+CD62L^+$ T cell-induced colitis. The protective effects of IFN-γ-MdCs on intestinal inflammation *in vivo* were speculated to depend on T cell-suppression since IFN-γ-MdCs *in vitro* deleted lymphocytes that had been isolated from mesenteric lymph nodes of colitic mice and enriched surviving lymphocytes for $CD4^+CD25^+Foxp3^+$ regulatory T cells (Brem-Exner et al., 2008). In another study, intravenous ($1x10^6$) as well as intraperitoneal ($0.75x10^6$) injection of peritoneal macrophages after *in vitro* treatment with IL-4/IL-13 (M2a activation) reduced intestinal inflammation in a murine model of DNBS-induced colitis in an IL-10-dependent manner (Hunter et al., 2010). Tierney and colleagues (2009) showed that *in vitro* treatment of

murine BMMΦ with LPS and immune complexes induces M2b macrophages which are able to prevent the development of EAE. In the respective study, $2x10^6$ M2b macrophages were intraperitoneally transferred into mice prior to induction of EAE with the MOG_{35-55} peptide. Compared to control groups, ConA-stimulated splenocytes of M2b macrophage-treated animals showed higher production of IL-10 suggesting that the suppressive effects on EAE were mediated through the induction of IL-10-producing T cells (Tierney et al. 2009).

Different studies found macrophages to exert suppressive and regenerative functions also in acute and chronic kidney diseases. Zhang et al. (2011) showed that binding of serum amyloid P component to activated lymphocyte-derived DNA (ALD-DNA) forms complexes which *in vitro* induces M2a activation of murine BMMΦ. Intravenous transfer of $2.5x10^6$ SAP/ALD-DNA-induced M2a macrophages for a total of three times successfully extenuated murine lupus nephritis through the production of IL-10 (Zhang et al., 2011). In another study, intravenous injection of $1x10^7$ untreated BMMΦ into mice ameliorated renal fibrosis in a model of late stage unilateral ureteral obstruction (UUO) that reflects progressive renal interstitial fibrosis. Three-time repeated transfer of BMMΦ was associated with an increased infiltration of interstitial macrophages which were suggested by the investigators to facilitate tissue repair and thereby attenuate renal fibrosis (Nishida et al., 2005). In a study by Cao et al. (2010), murine splenic macrophages were stimulated *in vitro* with IL-10 and TGF-β to induce M2c macrophage activation. Intravenous transfusion of $1x10^6$ IL-10/TGF-β-modified M2c macrophages was sufficient to mediate protection against renal injury in a murine model of adriamycin-induced nephropathy (AN). This protection was associated with an increase of $Foxp3^+$ regulatory T cells in renal draining lymph nodes. In a similar study, splenic macrophages were stimulated *in vitro* with IL-4/IL-13 and transferred into SCID mice. Transfer of these M2a-polarized macrophages reduced inflammatory renal disease possibly by affecting chemokine and cytokine expression (decrease of CCL2 and TNF-α and increase of CCL17) of infiltrating host macrophages (Wang et al., 2007). In a recent study by Jung et al. (2012), intravenous transfer of IL-10-overexpressing rat BMMΦ protected recipient rats against renal ischemia/reperfusion injury (IRI). After infusion of $1x10^6$ cells, macrophages migrated to the injured kidney tissue, increased regenerative markers such as stathmin, a protein that regulates the microtubule cytoskeleton (Rubin and Atweh, 2004), and proliferation cellular nuclear antigen (PCNA), a nuclear protein which is associated with the S phase of proliferation (Theocharis et al., 1994), and reduced blood urea nitrogen and creatinine. The macrophage-mediated protection was mediated via IL-10 and dependent on intracellular iron and lipocalin-2 as binding of iron through a chelating agent or the blockade of lipocalin-2 by a neutralizing antibody reversed the protective effects.

Transfer of macrophages can also protect against bacterial pneumonia. This was demonstrated in a study by Hart et al. (2003). The investigators intravenously and intraperitoneally transferred $2x10^7$ C2D macrophages into immunocompromised mice thereby protecting the animals against pneumonia after challenge with *Pasteurella pneumotropica*. The results of the present thesis further provide evidence that treatment with helminth immunomodulators can induce macrophages capable of mediating immunosuppressive effects in different disease contexts. Collectively, these results show that the option to modify macrophages towards a suppressive phenotype or to use specific macrophage cell lines for the treatment of diverse diseases constitutes a promising alternative to currently used forms of therapy.

original cells	treatment (phenotype)	application mode	amount of transferred cells	disease model	reference
human splenic TAICs	IFN-γ (IFN-γ-TAICs)	i.v.	$1.07x10^6$ / kg body weight	kidney transplantation	Hutchinson et al. (2009)
murine BMMΦ	IFN-γ (IFN-γ MdCs)	i.v.	$5x10^6$	$CD4^+CD62L^+$ T cell-induced colitis	Brem-Exner et al. (2008)
murine peritoneal MΦ	IL-4 and IL-13 (M2a)	i.v. and i.p.	$0.75x10^6$ (i.p.) $1x10^6$ (i.v.)	DNBS-colitis	Hunter et al. (2010)
murine BMMΦ	IFN-γ + LPS + IC (M2b)	i.p.	$2x10^6$	EAE	Tierney et al. (2009)
murine BMMΦ	SAP/ALD-DNA (M2a)	3 x i.v.	$2.5x10^6$	lupus nephritis	Zhang et al. (2011)
murine BMMΦ	no stimulation	3 x i.v.	$1x10^7$	UUO	Nishida et al. (2005)
murine splenic MΦ	IL-10 and TGF-β (M2c)	i.v.	$1x10^6$	AN	Cao et al. (2010)
murine splenic MΦ	IL-4 and IL-13 for (M2a)	i.v.	not stated	AN	Wang et al. (2007)
IL-10-overexpressing rat BMMΦ	no stimulation	i.v.	$1x10^6$	IRI	Jung et al. (2012)
murine C2D cell line	no stimulation	i.v. and i.p.	$2x10^7$	bacterial pneumonia	Hart et al. (2003)

Table 5-1: MΦ-based therapy in different disease settings. TAICs: transplant acceptance-inducing cells; MΦ: macrophage; BMMΦ: bone marrow-derived MΦ; IFN-γ MdCs: IFN-γ-stimulated monocyte-derived cells; SAP: serum amyloid P component; ALD-DNA: activated lymphocyte-derived DNA; i.p.: intraperitoneal; i.v.: intravenous; DNBS: dinitrobenzene sulfonic acid; EAE: experimental autoimmune encephalomyelitis; UUO: unilateral ureteral obstruction; AN: adriamycin nephropathy; IRI: renal ischemia/reperfusion injury.

6 OUTLOOK

Since the inhibition of distinct macrophage markers (SPHK-1, ARG-1, PD-L1 and PD-L2) did not affect IL-10-induction in $CD4^+$ T cells, it is necessary to identify additional products (proteins, lipids, enzymes etc.) secreted by AvCystatin-MΦ which may be responsible for this effect. This might include a combination of different techniques such as microarray analysis for a broader screen of AvCystatin-induced gene expression in macrophages, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and mass spectrometry techniques. Studies on the stability of identified factors will show whether they can be synthesized on a large scale and used to generate immunosuppressive $CD4^+$ T cells. In parallel, it is important to unravel the receptors on and signalling pathways in $CD4^+$ T cells addressed by the macrophage-secreted mediator(s). This could either be realized through the inhibition of specific receptors and signalling pathways that lead to IL-10 induction in $CD4^+$ T cells or alternatively by subtractive gene expression analysis of $CD4^+$ T cells treated with supernatants from AvCystatin- or control-stimulated macrophages.

Another interesting question will be whether the IL-10-inducing capacity of AvCystatin-MΦ depends on the macrophage phenotype. Although single marker genes were determined not to be directly involved in inducing this effect, a combination of these or other as yet undefined markers may be necessary for equipping macrophages with important immunosuppressive functions. Thus, it is advisable to examine the stability of the macrophage phenotype in more detail and to investigate whether the ability of macrophages to induce IL-10-producing $CD4^+$ T cells is affected by modified environmental conditions. Concerning the therapeutic usability of AvCystatin-MΦ, it would be of great advantage if (a) the macrophages perpetuate their phenotype and/or suppressive functions after having been frozen and (b) the macrophages could be increased in numbers through *in vitro* treatment with growth factors. The latter scenario seems to be realistic since the ability of macrophages to proliferate has recently been reported (Jenkins et al., 2011).

In regard to the model of ovalbumin-induced airway hyerreactivity it will be worth to study whether AvCystatin-MΦ are still protective if mice are challenged at a later timepoint after transfer of macrophages or alternatively if a time-delayed second challenge is given. This would reflect the situation of allergic patients who are seasonally exposed to allergens. In the present study, AvCystatin-MΦ were transferred after *in vivo* stimulation for 18-20 hours. To examine whether AvCystatin-MΦ exert prolonged suppressive activity, experiments have to be performed in which AvCystatin-MΦ are transferred into mice at later timepoints (e.g. 48 hours, 72 hours, 96 hours etc. after contact with AvCystatin).

The mechanism of the AvCystatin-MΦ-mediated immunosuppression in DSS-induced colitis remains elusive. Compared to the ovalbumin-induced model of airway hyperreactivity, no induction of IL-10-producing T cells was determined in recipient mice after administration of AvCystatin-MΦ. Hence, an important aspect of continuing investigations is to elucidate functional differences of AvCystatin-MΦ in the respective disease models. It seems to be cogitable that AvCystatin-MΦ either directly modulate the cytokine and chemokine response of resident host macrophages or mediate protection against intestinal inflammation by affecting the chemokine expression of epithelial cells or tight junction functions.

Another feature for ongoing projects is the idea to test AvCystatin-MΦ in a model of helminth infection thereby evaluating if the macrophages suppress Th2 immune responses and mediate parasite survival or rather promote host protection and worm expulsion. Since AvCystatin-MΦ also express markers which have been shown to exert important functions in other disease settings, it might be worth testing AvCystatin-MΦ in corresponding contexts. ICAM-1, for example, was found to induce apoptosis in tumor cells (Zhai et al., 1998; Rooney et al., 2000). Thus, the effects of AvCystatin-MΦ on cancer cells could be tested by using a murine or human tumor cell line. Preliminary data further show AvCystatin-MΦ to up-regulate lipocalin-2, a 25 kDa protein that has been reported to mediate protection against ischemia/reperfusion injury (Jung et al., 2012). If lipocalin-2 expression in AvCystatin-MΦ is repeatable, it will also be useful to evaluate AvCystatin-MΦ functions in a disease model of kidney disease.

To unlock clinical benefits of macrophages, it is essential to translate experimental findings from mouse studies to the human situation. Thus, studying the key molecular events for the induction of suppressive human macrophages by AvCystatin is a major concern for future projects. However, while several activation markers have been described for murine macrophages, there is still an obvious lack of reliable human markers. ARG-1 or Ym1, for example, are restricted to murine M2 macrophages and thus constitute improper markers for human alternatively activated myeloid cells (Raes et al., 2005). One general problem in translating data from mouse macrophages to human cells arises due to the fact that human macrophages respond differently to Th2 cytokines (Scotton et al., 2005). Another difficulty for translational research is that relevant human tissues are not as easily accessible for experimental investigation as they are for mice (Jenkins and Allen, 2010). To study whether human monocyte-derived macrophages show phenotypic properties or regulatory functions similar to AvCystatin-MΦ, constitutes an important step towards the clinics and will show whether findings from the present investigation can be translated into the human system.

7 MATERIAL AND METHODS

7.1 Material

7.1.1 Laboratory equipment

accu-jet pro pipette	Amersham Pharmacia, Freiburg, Germany
autoMACS cell sorter	Miltenyi Biotec, Bergisch Gladbach, Germany
Casy cell counter	Innovatis, Reutlingen, Germany
cell incubator	Heraeus, Hanau, Germany
centrifuge 5804 R	Eppendorf, Hamburg, Germany
cytospin centrifuge	Cellspin I, Tharmac, Waldsolms, Germany
DFC480 camera	Leica, Solms, Germany
electrophoresis power supply	Amersham Pharmacia, Freiburg, Germany
ELISA reader	Dynatech, Denkendorf, Germany
ELx405 select ELISA washer	BioTek, Bad Friedrichshall, Germany
FastPrep-24 homogenizer	MP Biomedicals, Heidelberg, Germany
FPLC ÄKTA	Amersham Pharmacia, Freiburg, Germany
Hera Safe sterile work bench	Heraeus Instruments, Osterode, Germany
Leica DFC480 camera	Leica, Solms, Germany
Leica DM500 microscope	Leica, Solms, Germany
Leica TCS SP2 confocal microscope	Leica, Solms, Germany
LSRII Fortessa flow cytometer	BD Biosystems, Heidelberg, Germany
magnetic stirrer (RCT Basic)	IKA, Staufen, Germany
NanoDrop ND-1000	Peqlab Biotechnologie, Erlangen, Germany
real-time PCR system 7300	Applied Biosystems, Darmstadt, Germany
semi-dry electroblotting system	Peqlab Biotechnologie, Erlangen, Germany
thermocycler (FlexCycler)	Analytikjena, Jena, Germany
thermomixer comfort thermoblock	Eppendorf, Hamburg, Germany
waterbath DC3	Haake, Karlsruhe, Germany

7.1.2 Consumables

1 ml tuberculin single use syringe	Braun, Melsungen, Germany
1 ml U-40 single use insulin syringe	Terumo, Eschborn, Germany
1.0 µm thin certs 24 well	greiner bio-one, Frickenhausen, Germany
24 well culture cluster	Corning Costar, Bodenheim, Germany
48 well culture cluster	Corning Costar, Bodenheim, Germany
96 well culture cluster	Corning Costar, Bodenheim, Germany
96 well flat bottom ELISA plate	ThermoFisher Scientific, Schwerte, Germany
96 well multiply-PCR plate	Sarstedt, Nümbrecht, Germany
biosphere filter tips (200 µl)	Sarstedt, Nümbrecht, Germany
C-Chip disposable hemocytometer	Digital Bio, Seoul, Korea
cell strainer (70 µm Nylon)	BD Biosciences, Heidelberg, Germany
cryo tubes (CRYO.S)	greiner bio-one, Frickenhausen, Germany
EndoTrap red columns	Profos, Regensburg, Germany
eppendorf tubes (1.5 and 2 ml)	Eppendorf, Hamburg, Germany
falcon cellstar tubes (15 and 50 ml)	greiner bio-one, Frickenhausen, Germany
FastPrep-24 lysing matrix tubes D	MP Biomedicals, Heidelberg, Germany
gauze swabs	Paul Hartmann, Heidenheim, Germany
hypodermic needle (0.4x20 mm)	Braun, Melsungen, Germany
MACS pre-separation filter (30 µm)	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS separation columns	Miltenyi Biotec, Bergisch Gladbach, Germany
microscope slides (76x26 mm)	ThermoFisher Scientific, Schwerte, Germany
microtainer brand tubes	BD Biosciences, Heidelberg, Germany
PCR soft tubes (0.2 ml)	Biozym, Hessisch Oldendorf, Germany
petri dishes (21x16 mm)	Sarstedt, Nümbrecht, Germany
safe seal-tips (10 µl und 1000 µl)	Biozym, Hessisch Oldendorf, Germany
sealing tape for 96 well PCR plate	Sarstedt, Nümbrecht, Germany
single use filter (0.20 and 0.45 µm)	Sartorius Stedim Biotech, Goettingen, Germany
single use syringe (5 and 10 ml)	Braun, Melsungen, Germany

stripette (10, 25 and 50 ml)	Corning Costar, Bodenheim, Germany
titertube micro tubes	BIO-RAD, München, Germany
tracheal drain tubes (0.7x19 mm)	BD Biosciences, Heidelberg, Germany
transfer pipettes (3.5 ml)	Sarstedt, Nümbrecht, Germany
U96 maxisorp NUNC-immuno plate	Nunc, Wiesbaden, Germany
Whatman blotting papers	Schleicher & Schuell, Dassel, Germany

7.1.3 Buffer, media and staining solutions

Cell culture and cell preparation

DMEM complete (cDMEM)	DMEM high glucose (PAA, Cölbe, Germany) 10% (v/v) fetal bovine serum 1 mM L-glutamine 100 U/ml penicillin G 100 mg/ml streptomycin
RPMI complete (cRPMI)	RPMI (PAA, Cölbe, Germany) 10% (v/v) fetal bovine serum 1 mM L-glutamine 100 U/ml penicillin G 100 mg/ml streptomycin
phosphate buffered saline (PBS)	140 mM NaCl 3.4 mM KCl 1.8 mM KH_2PO_4 10 mM Na_2HPO_4
erythrocyte lysis buffer (pH 7.5)	0.02 M $KHCO_3$ 0.155 M NH_4Cl 0.1 mM EDTA
intestinal wash buffer	Hank`s BBS (PAA, Cölbe, Germany) 0.1% (v/v) bovine serum albumin (BSA)

Cell sorting and flow cytometry

FACS buffer	PBS, 0.2% (v/v) BSA, 2 mM EDTA
running buffer (MACS)	PBS, 0.2% (v/v) BSA, 2 mM EDTA
rinsing buffer (MACS)	PBS, 2 mM EDTA

Protein purification (AvCystatin and SNAP-tag)

LB medium	10 g Tryptone
	10 g NaCl
	5 g yeast extract
	ad. 1 liter H_2O dest.
lysis buffer A1	PBS, 0.1% (v/v) Triton X-100, pH 8
wash buffer 1	PBS, 10% (v/v) glycerin, pH 6
wash buffer 2	PBS, 10% (v/v) glycerin, pH 5
elution buffer	PBS, 10% (v/v) glycerin, pH 3
neutralization buffer	PBS, pH 10
dialysis buffer	PBS, pH 7.4

Activity test

papain reaction buffer	0.2 M K_2HPO_4
	0.2 M KH_2PO_4
	0.004 M DTT
	0.004 M EDTA
	0.01% (v/v) Brij 35

SDS-PAGE

2x loading buffer	10% (v/v) glycerol
	5% (v/v) beta-mercaptoethanol
	3% (v/v) SDS
	100 mM Tris-HCl
buffer L (pH 8.4)	1.5 Tris-HCl, 0.4% (v/v) SDS
buffer M (pH 6.8)	0.5 M Tris HCl, 0.4% (v/v) SDS
stacking gel (6%)	0.75 ml acrylamide (rotiphorese gel 30)
	1.25 ml buffer M
	5 µl TEMED
	3 ml H_2O bidest.
	50 µl APS (10%)
separation gel (14%)	4.7 ml acrylamide (rotiphorese gel 30)
	2.5 ml buffer L
	10 µl TEMED
	2.8 ml H_2O bidest.
	100 µl APS (10%)
SDS running buffer	250 mM Tris-HCl
	1.9 M glycine
	1% (v/v) SDS
Coomassie staining	2 tablets PhastGel blue R
	20% (v/v) ethanol
	10% (v/v) acetic acid

destaining solution	20% (v/v) ethanol
	10% (v/v) acetic acid

Western blot analysis

cell lysis buffer	10 mM Tris-HCl, pH 7.2
	1% (v/v) DOC
	1% (v/v) Triton X-100
	1 mM PMSF
	150 mM NaCl
	50 µg/ml leupeptin
	1 mM Na-orthovanadate
	50 mM NaF
transfer buffer	48 mM Tris-HCl
	39 mM glycine
	0.037% (v/v) SDS
	20% (v/v) methanol
TBST	10 mM Tris pH 8.0
	150 mM NaCl
	0.1% (v/v) Tween 20

7.1.4 Chemicals and biological reagents

7.1.4.1 Chemicals

bovine serum albumin fraction V	AppliChem, Darmstadt, Germany
Bz-Arg-pNA (BAPNA)	Bachem, Bubendorf, Germany
dextran sodium sulfate	MP Biomedicals, Heidelberg, Germany
EDTA	AppliChem, Darmstadt, Germany
ethanol (absolute)	AppliChem, Darmstadt, Germany
fetal bovine serum	Biochrom, Berlin, Germany
Imject Alum adjuvant	ThermoFischer Scientific, Schwerte, Germany
LAL reagent water	Lonza, Cologne, Germany
L-glutamine	PAA Laboratories, Cölbe, Germany
lysozyme	Sigma-Aldrich, Steinheim, Germany
nonfat dried milk powder	AppliChem, Darmstadt, Germany
ovalbumin from chicken egg	Sigma-Aldrich, Steinheim, Germany
papain from *Carica papaya*	Sigma-Aldrich, Steinheim, Germany
paraformaldehyde	Carl Roth, Karlsruhe, Germany
penicillin/streptomycin	PAA Laboratories, Cölbe, Germany
Percoll	GE Healthcare, München, Germany
PhastGel blue R tablets	Amersham Pharmacia, Freiburg, Germany
phosphate-citrate buffer tablets	Sigma-Aldrich, Steinheim, Germany
rotiphorese gel 30 (acrylamid)	Carl Roth, Karlsruhe, Germany
sphingosine-1-phosphate (S1P)	Calbiochem/Merck, Darmstadt, Germany
TMB tablets	Sigma-Aldrich, Steinheim, Germany
water (molecular biology grade)	AppliChem, Darmstadt, Germany

7.1.4.2 MicroBeads and antibodies

MicroBeads, dyes and antibodies which were used for cell purification, flow cytometry, western blot analysis and subclass-specific ELISA are listed in the following table.

antibody	conjugate	clone/source	manufacturer
anti-AKT	-	rabbit	Cell Signalling
anti-AvCystatin	-	rabbit	Dianova
anti-Biotin MicroBeads	-	-	Miltenyi Biotec
anti-DUSP-1	-	rabbit	Santa Cruz
anti-IL-10 receptor	-	1B1	DRFZ
anti-mouse CD273	-	TY25	eBioscience
anti-mouse CD274	-	MIH5	eBioscience
anti-mouse CD4	PerCp-eF710	RM4-5	eBioscience
anti-mouse Foxp3	PE	FKJ-16s	eBioscience
anti-mouse IFN-γ	Pacific Blue	XMG1.2	eBioscience
anti-mouse IgE	-	R35-72	BD Biosciences
anti-mouse IgE	HRP	MCA419P	AbD Serotec
anti-mouse IgE, κ	-	C38-2	BD Biosciences
anti-mouse IgG1	AP	Polyclonal	Rockland Immunochemicals
anti-mouse IgG1, κ	-	MOPC-31C	BD Biosciences
anti-mouse IgG2a	AP	Polyclonal	Rockland Immunochemicals
anti-mouse IgG2a, κ	-	G155-178	BD Biosciences
anti-mouse IL-10	PE	JES5-16E3	BD Biosciences
anti-mouse-CD11b	FITC	M1/70	BD Biosciences
anti-mouse-CD11c	Biotin	N418	eBioscience
anti-mouse-CD11c	Pacific Blue	N418	BD Biosciences
anti-mouse-CD16/32	APC	2.4G2	DRFZ
anti-mouse-CD19	Biotin	eBio1D3	eBioscience
anti-mouse-CD19	FITC	1D3	eBioscience
anti-mouse-CD273	PE	TY25	BD Biosciences
anti-mouse-CD274	PE	MIH5	BD Biosciences
anti-mouse-CD3ε	Biotin	145-2C11	Miltenyi Biotec
anti-mouse-CD4	Pacific Blue	RM4-5	BD Biosciences
anti-mouse-CD40	PE	1C10	eBioscience
anti-mouse-CD80	PE	16-10A1	eBioscience

anti-mouse-CD86	Biotin	GL1	eBioscience
anti-mouse-Dx5	Biotin	Dx5	eBioscience
anti-mouse-F4/80	APC	BM8	eBioscience
anti-mouse-FcγR	-	2.4G2	DRFZ
anti-mouse-Gr1	Biotin	RB6-8C5	DRFZ
anti-mouse-ICAM-1	APC	KAT1	DRFZ
anti-mouse-MHC-II	FITC	M5/114.15.2	eBioscience
anti-ovalbumin IgG	-	rabbit	Acris Antibodies
anti-p38 MAPK	-	rabbit	Cell Signalling
anti-p44/42 MAPK	-	rabbit	Cell Signalling
anti-phospho-AKT (Ser473)	-	rabbit	Cell Signalling
anti-phospho-DUSP-1 (Ser359)	-	rabbit	Cell Signalling
anti-phospho-p38 MAPK (Thr180/Tyr182)	-	rabbit	Cell Signalling
anti-phospho-p44/42 MAPK (Thr202/Tyr204)	-	rabbit	Cell Signalling
anti-β-actin	-	rabbit	Cell Signalling
armenian hamster IgE isotype control	PECy7	HTK888	BioLegend
CD19 MicroBeads	-	-	Miltenyi Biotec
CD25	APC	PC61	BD Biosciences
DyLight549	-	-	ThermoFisher Scientific
fixable cell viability dye	eF780	-	eBioscience
rat IgG1 isotype control	PE	eBRG1	eBioscience
rat IgG1 isotype control	APC	eBRG1	eBioscience
rat IgG1 isotype control	FITC	eBRG1	eBioscience
rat IgG1 isotype control	biotin	eBRG1	eBioscience
rat IgG2b κ isotype control	PerCp-eF710	eB149/10H5	eBioscience
streptavidin	PECy7	Streptavidin	BD Biosciences

7.1.4.3 Cytokines

murine GM-CSF	PreproTech, Hamburg, Germany
murine IFN-γ	PreproTech, Hamburg, Germany
murine IL-4	PreproTech, Hamburg, Germany
murine IL-13	PreproTech, Hamburg, Germany
murine IL-21	PreproTech, Hamburg, Germany

7.1.4.4 Inhibitors and antagonists

BEC (arginase-1-inhibitor)	Calbiochem/Merck, Darmstadt, Germany
cytochalasin D	Sigma-Aldrich, Steinheim, Germany
genistein (tyrosine kinase-inhibitor)	Calbiochem/Merck, Darmstadt, Germany
JNK inhibitor II (JNK-inhibitor)	Calbiochem/Merck, Darmstadt, Germany
Ly294002 (PI3K-inhibitor)	Calbiochem/Merck, Darmstadt, Germany
proteinase inhibitor cocktail	Roche, Mannheim, Germany
SB203580 (p38-inhibitor)	Calbiochem/Merck, Darmstadt, Germany
U0126 (MEK1/2-inhibitor)	Calbiochem/Merck, Darmstadt, Germany
VPC23019 (S1PR1 antagonist)	Avanti Polar Lipids, Alabaster, USA

7.1.4.5 Other reagents

brefeldin A (BFA)	Sigma-Aldrich, Steinheim, Germany
CFSE	Sigma-Aldrich, Steinheim, Germany
collagenase VIII	Sigma-Aldrich, Steinheim, Germany
concanavalin A	GE Healthcare, München, Germany
DNA-ExitusPlus	AppliChem, Darmstadt, Germany
ionomycin	Sigma-Aldrich, Steinheim, Germany
isoflurane	Baxter, Munich, Germany
lipopolysaccharide	Sigma-Aldrich, Steinheim, Germany
Ni-NTA agarose	Qiagen, Hilden, Germany

OVA-peptide 323-339	GenScript, Piscataway, USA
phorbol-12-myristate-acetate (PMA)	Sigma-Aldrich, Steinheim, Germany
RNA stabilization reagent (RNAlater)	Qiagen, Hilden, Germany
RNase-ExitusPlus	AppliChem, Darmstadt, Germany

7.1.5 Primer sequences

Primer pairs for quantitative real-time PCR were synthesised by and purchased from either TIB-MOLBIOL (Berlin, Germany) [1] or RealTimePrimers.com (Pennsylvania, USA) [2].

gene	accession number	sequence (5´- 3´)
Arginase-1 [1]	NM_007482	fw: CAGAAGAATGGAAGAGTCAG rv: CAGATATGCAGGGAGTCACC
CCL1 [2]	NM_011329	fw: TGCTTACGGTCTCCAATAGC rv: TTTTGAACCCACGTTTTGTT
CCR3 [1]	NM_009914	fw: TCAACTTGGCAATTTCTGACCT rv: CAGCATGGACGATAGCCAGG
DUSP-1 [1]	NM_013642	fw: ACAACAATGACTTGACCGCA rv: GGAATGGTTAATACTGGTGG
DUSP-10 [2]	NM_022019	fw: CGCCTACTTGATGAAGCACA rv: AGGTTCGGGGAAATAATTGG
DUSP-2 [2]	NM_010090	fw: CAAGGGCTCAGAAAACAAGC rv: CAGCACCAATTACAGCGAGA
DUSP-3 [1]	NM_028207	fw: TCTGTGGCTCAGGACATCAC rv: GGCCCTTTCAAAGTAAGCACTG
DUSP-5 [2]	NM_001085390	fw: TGCACCACCCACCTACACTA rv: CATGAGGTAAGCCATGCAGA
DUSP-6 [2]	NM_026268	fw: GGCAAAAACTGTGGTGTCCT rv: CATCGTTCATGGACAGGTTG
Eotaxin-1 [1]	NM_011330	fw: GAATCACCAACAACAGATGCAC rv: ATCCTGGACCCACTTCTTCTT
Eotaxin-2 [1]	NM_019577	fw: ATTCTGTGACCATCCCCTCAT rv: TGTATGTGCCTCTGAACCCAC
IL-10 [1]	NM_010548	fw: GCTCTTACTGACTGGCATGAG rv: CGCAGCTCTAGGAGCATGTG
IL-12/23p40 [1]	NM_008352	fw: ATGGCCATGTGGGAGCTGGAGAAAG rv: GTGGAGCAGCAGATGTGAGTGGCT
IL-6 [2]	NM_031168	fw: AGTTGCCTTCTTGGGACTGA rv: TCCACGATTTCCCAGAGAAC
iNOS [1]	NM_010927	fw: CCCTTCCGAAGTTTCTGGCAGCAGC rv: GGCTGTCAGAGCCTCGTGGCTTTGG

LIGHT [1]	NM_019418	fw: CTGCATCAACGTCTTGGAGA rv: GATACGTCAAGCCCCTCAAG
MCP-1 [1]	NM_011333	fw: TTAAAAACCTGGATCGGAACCAA rv: GCATTAGCTTCAGATTTACGGGT
MCP-5 [1]	NM_011331	fw: ATTTCCACACTTCTATGCCTCCT rv: ATCCAGTATGGTCCTGAAGATCA
PPIA [1]	NM_008907	fw: GAGCTGTTTGCAGACAAAGTTC rv: CCCTGGCACATGAATCCTGG
RANTES [1]	NM_013653	fw: GCTGCTTTGCCTACCTCTCC rv: TCGAGTGACAAACACGACTGC
RELM-α [1]	NM_020509	fw: TCCCAGTGAATACTGATGAGA rv: CCACTCTGGATCTCCCAAGA
SPHK-1 [1]	NM_011451	fw: GGCAGTCATGTCCGGTGATG rv: ACAGCAGTGTGCAGTTGATGA
TGF-β [2]	NM_011577	fw: GCTACCATGCCAACTTCTGT rv: CGTAGTAGACGATGGGCAGT
TNF-α [1]	NM_013693	fw: CCACGTCGTAGCAAACCAC rv: TGGGTGAGGAGCACGTAGT
VCAM-1 [1]	NM_011693	fw: AGTTGGGGATTCGGTTGTTCT rv: CCCCTCATTCCTTACCACCC
Ym1 [1]	NM_009892	fw: CATGAGCAAGACTTGCGTGAC rv: GGTCCAAACTTCCATCCTCCA
Beta-actin [1]	NM_007393	fw: GGCTGTATTCCCCTCCATCG rv: CCAGTTGGTAACAATGCCATGT

7.1.6 Commercial kits

BCA protein assay kit	Pierce Biotechnology, Rockford, USA
$CD4^+$ T cell isolation kit, mouse	Miltenyi Biotec, Bergisch Gladbach, Germay
ECL plus detection system	Amersham Pharmacia, Freiburg, Germany
EndoTrap red 10	Profos, Regensburg, Germany
FastStart universal SYBR green	Roche, Mannheim, Germany
Hemacolor staining set	Merck, Darmstadt, Germany
High Capacity RNA-to-cDNA kit	Applied Biosystems, Darmstadt, Germany
innuPREP RNA kit	Analytikjena, Jena, Germany
Limulus amoebocyte lysate QCL 1000	Cambrex, Walkersville, USA
mouse IFN-γ ELISA Ready-Set-Go	eBioscience, Frankfurt am Main, Germany
mouse IL-10 OptEIA ELISA set	BD Biosciences, Heidelberg, Germany
mouse IL-12 (p40) OptEIA ELISA set	BD Biosciences, Heidelberg, Germany
mouse IL-13 ELISA Ready-Set-Go	eBioscience, Frankfurt am Main, Germany
mouse IL-2 ELISA Ready-Set-Go	eBioscience, Frankfurt am Main, Germany
mouse IL-4 ELISA Ready-Set-Go	eBioscience, Frankfurt am Main, Germany
mouse IL-5 ELISA Ready-Set-go	eBioscience, Frankfurt am Main, Germany
QuantiChrom urea assay kit	BioAssay Systems, Hayward, USA

7.1.7 Software

EndNote (v. X4.0)	Thomson Reuters, Carlsbad, USA
Adobe Photoshop CS3 (v. 10.0)	Adobe Systems Incorporated, San Jose, USA
Application Suite Software (v. 2.8.1)	Leica, Solms, Germany
FlowJo Software (v. 8.8.7)	Tree Star, Ashland, USA
GraphPad Prism Software (v. 5.02)	GraphPad Software, San Diego, USA

7.2 Methods

7.2.1 Animals and ethics statement

If not otherwise stated animals were purchased from Charles River, Sulzfeld, Germany or the Federal Institute for Risk Assessement (BfR) in Berlin, Germany. DUSP-1-deficient mice and control littermates were a kind gift from Roland Lang, Friedrich-Alexander Universität, Erlangen-Nürnberg, Germany. IL-10-deficient mice were provided by Ute Hoffmann and Alf Hamann, German Rheumatism Research Centre (DRFZ), Charité Hospital, Berlin, Germany. All experiments were approved by the animal ethics committee of the local government (State Office of Health and Social Affairs Berlin, Germany) and performed in accordance with the German law and national guidelines for animal protection. Animals were kept in ICV-cages with filter caps and sacrificed with isoflurane (Baxter, Munich, Germany).

7.2.2 Basic laboratory techniques

7.2.2.1 Protein purification

AvCystatin and SNAP-tag control protein were expressed in *Escherichia coli*. Pellets were resuspendet in lysis buffer (PBS, 0.1% Triton, pH 8) and incubated with lysozyme (Sigma-Aldrich, Steinheim, Germany) on ice for 30 minutes. The cell suspension was sonificated (30 cycles, MS72/D for 2 minutes) and centrifuged with 12000 rpm at 4°C for 15 minutes. Supernatants were collected and purified by affinity chromatography using Ni-NTA agarose (Qiagen, Hilden, Germany). Proteins were eluted by pH gradient (consecutive treatment with PBS, 10% glycerin, pH 6, 5 and 3), neutralized by neutralization buffer (PBS, pH 10) and afterwards dialysed against PBS over night and for additional two hours on the following day. Endotoxin was removed with EndoTrap red columns (Profos, Regensburg, Germany) and final concentrations measured by Limulus amoebocyte lysate (LAL) Kit QCL 1000 (Cambrex, Walkersville, USA). Residual endotoxin levels of purified proteins were below 0.1 endotoxin units/ml (EU/ml). Protein concentrations were measured by BCA protein assay kit according to the manufacturer`s instructions (Pierce Biotechnology, Rockford, USA). AvCystatin was heat-inactivated by cooking at 95°C for 10 minutes and used as a control to exclude that modulatory effects on macrophages arise from contaminations in the buffer. The control protein SNAP-tag constitutes a 20 kDa mutant of the DNA repair protein O6-alkylguanine-DNA alkyltransferase and does not exert side effects on macrophage cultures as determined by own experiments (data not shown). Both controls, denatured AvCystatin and SNAP-tag protein, were determined to have similar residual LPS values as AvCystatin protein.

7.2.2.2 Activity test

To test the inhibitory activity of AvCystatin, purified protein was incubated in a dose-dependent manner with the cysteine protease papain (Sigma-Aldrich, Steinheim, Germany) and its substrate Bz-Arg-pNA (Bachem, Bubendorf, Switzerland) in a 96 well flat bottom ELISA plate (ThermoFisher Scientific, Schwerte, Germany). Papain was solved in reaction buffer (0.2 M K_2HPO_4, 0.2 M KH_2PO_4, 0.004 M DTT, 0.004 M EDTA and 0.01% Brij 35) for a concentration of 100 µM. Bz-Arg-pNA (BAPNA) was solved in DMSO for a concentration of 92 µM. 5 µl papain solution (100 µM) was incubated with 10 µl BAPNA (92 µM), different concentrations of AvCystatin (0.05 µM, 0.1 µM, 0.25 µM, 0.5 µM, 1 µM, 2 µM and 3 µM) and the respective amount PBS for a total volume of 200 µl per well. Plates were incubated for 30 minutes at 37°C and the substrate absorption measured at a wavelength of 405 nm.

7.2.2.3 SDS-PAGE and Coomassie staining

In order to determine protein expression or to perform western blot analysis, proteins were separated through sodium-dodecyl-sulfate-polyacrylamid-gelelectrophoresis (SDS-PAGE). Total cell extracts were mixed with 2x sample buffer and heated for 5 minutes at 95°C. Gels of 14% polyacrylamid were used in combination with a 6% stacking gel and run at 120V/400mA. Coomassie staining (2 PhastGel blue R tablets dissolved in 20% ethanol and 10% acetic acid) was performed for 30 minutes at 60°C. Gels were thereafter treated with destaining solution (20% ethanol, 10% acetic acid) to remove unbound Coomassie.

7.2.2.4 Western blot analysis

Cells were treated with lysis buffer (10 mM Tris-HCl pH 7.2, 1% DOC, 1% Triton X-100, 1 mM PMSF, 150 mM NaCl, 50 µg/ml leupeptin, 1 mM Na-orthovanadate, 50 mM NaF) and 7-30 µg cell extract separated by SDS-PAGE. After blotting, Whatman membranes (Schleicher & Schuell, Dassel, Germany) were blocked in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20) with 5% non-fat dry milk (AppliChem, Darmstadt, Germany) for one hour and incubated with primary antibodies overnight. On the following day, membranes were washed, incubated with secondary peroxidase-conjugated antibodies and signals detected via chemiluminescence reaction by using the ECL plus western blotting detection system according to the manufacturer`s instructions (Amersham Pharmacia Biotech, Freiburg, Germany).

7.2.2.5 RNA isolation, reverse transcription and real-time PCR

Isolation of total RNA and reverse transcription was performed with the innuPREP RNA kit (Analytikjena, Jena, Germany) and the High Capacity RNA-to-cDNA kit (Applied Biosystems, Darmstadt, Germany). Real-time PCR was performed with the ABI Prism 7300 sequence detection system (Applied Biosystems, Darmstadt, Germany) by using SYBR Green PCR reagents (FastStart Universal SYBR Green Master (Rox)) according to the manufacturer`s guidelines (Roche, Mannheim, Germany). Relative changes in gene expression were calculated with the ABI 7300 SDS software (Applied Biosystems, Darmstadt, Germany) and transcript levels normalized to ct values of the housekeeping gene peptidylprolyl isomerase A (PPIA) using the ΔΔct method (Livak and Schmittgen, 2001). PCR programme: 95°C for 10 minutes (1 cycle); 95°C for 15 seconds and 60°C for 30 seconds (40 cycles); 95°C for 15 seconds, 60°C for 30 seconds and 95°C for 15 seconds (1 cycle).

7.2.3 AvCystatin labeling and tracking

7.2.3.1 Uptake of AvCystatin by peritoneal cells

Recombinant AvCystatin was labelled with DyLight549 (ThermoFischer Scientific, Schwerte, Germany; labeling degree of 1 mole dye/mole AvCystatin) and intraperitoneally administered into BALB/c mice (40 µg/mouse). Peritoneal exudate cells were isolated 20 minutes after intraperitoneal injection and washed twice with cold FACS buffer (Dulbecco`s PBS, 0.2% BSA, 2 mM EDTA). PEC were stained with anti-CD4-Pacific Blue (clone RM4-5; BD Biosciences, Heidelberg, Germany), anti-CD19-FITC (clone 1D3; eBioscience, Frankfurt am Main, Germany), anti-F4/80-APC (clone BM8; eBioscience, Frankfurt am Main, Germany), anti-CD11b-FITC (clone M1/70; BD Biosciences, Heidelberg, Germany), anti-CD11c-Pacific Blue (clone N418; BD Biosciences, Heidelberg, Germany) and anti-Gr1-Biotin (clone RB6-8C5; DRFZ, Berlin, Germany), streptavidin-PECy7 (clone Streptavidin; BD Biosciences, Heidelberg, Germany). 20 µg/ml anti-mouse-FcγR (clone 2.4G2; DRFZ, Berlin, Germany) was added to prevent non-specific binding. Uptake of labelled AvCystatin was detected by LSRFortessa flow cytometer (BD Biosciences, Heidelberg, Germany) and analysed via FlowJo software version 8.8.7 (Tree Star, Ashland, USA). Moreover, PEC were isolated from BALB/c mice 20 minutes after intraperitoneal injection of DyLight594-labelled AvCystatin (40 µg/animal). Cells were washed with cold FACS buffer, treated with 20 µg/ml anti-mouse-FcγR (clone 2.4G2; DRFZ, Berlin, Germany) and stained with anti-CD11b-FITC (clone M1/70; BD Biosciences, Heidelberg, Germany). Confocal microscopy was performed by using Leica TCS SP2 confocal microscope (Leica, Solms, Germany).

7.2.3.2 Mode of AvCystatin uptake

PEC from BALB/c mice were incubated *in vitro* with 5 µg/ml DyLight549-AvCystatin for 30 minutes at 37°C or 4°C. After 30 minutes, cells were washed with cold FACS buffer, treated with 20 µg/ml anti-mouse-FcγR (clone 2.4G2; DRFZ, Berlin, Germany), stained with anti-F4/80-APC (clone BM8; eBioscience, Frankfurt am Main, Germany) and DyLight549 signals measured by flow cytometry. The kinetics of AvCystatin uptake was performed by western blot analysis. $2x10^6$ PEC of BALB/c mice were seeded in 24 well plates (Corning Costar, Bodenheim, Germany) and cultured in DMEM containing 1 mM L-glutamine, 100 U/ml penicillin G and 100 mg/ml streptomycin without FCS. After two hours, cells were washed with fresh and pre-warmed medium to remove non-adherend cells. Remaining cells were stimulated with 5 µg/ml AvCystatin at 37°C for various timepoints or at 4°C for 30 minutes. In addition, some cells were treated with AvCystatin in the presence of 10 mM cytochalasin D (Sigma-Aldrich, Steinheim, Germany) at 37°C for 15, 30 and 60 minutes. After indicated timepoints, cells were washed with cold Dulbecco`s PBS, treated with cell lysis buffer and whole cell extracts analysed by western blotting using primary antibodies against AvCystatin (Dianova, Hamburg, Germany) and β-actin (Cell Signalling Technology, Frankfurt am Main, Germany).

7.2.4 Detection of signalling events in AvCystatin-MΦ

7.2.4.1 Inhibitor studies

Peritoneal macrophages of naïve BALB/c mice were incubated *in vitro* with specific inhibitors (all inhibitors were tested in advance for cytotoxicity) 60 minutes prior to stimulation with 0.5 µM AvCystatin or control treatments (denatured AvCystatin or SNAP-tag). Briefly, macrophages were cultured with AvCystatin or control applications for 18 hours in the presence of PI3K inhibitor (Ly294002), p38 inhibitor (SB203580), MEK1/2 inhibitor (U0126), JNK inhibitor (JNK inhibitor II) and tyrosine kinase inhibitor genistein (all from Calbiochem/Merck, Darmstadt, Germany) in a dose-dependent manner. Culture supernatants were collected after 18 hours and analysed for IL-10 by ELISA according to the manufacturer`s instructions (BD Biosciences, Heidelberg, Germany).

7.2.4.2 Detection of MAPK phosphorylation in AvCystatin-MΦ

Peritoneal macrophages of BALB/c mice were stimulated *in vitro* for various timepoints with AvCystatin or denatured AvCystatin. The cells were washed afterwards with Dulbecco`s PBS and treated with cell lysis buffer. Western blot analysis of total cell extracts was performed by

using primary antibodies against phospho-p38 (Thr180/Tyr182), total p38, phospho-p44/42 (Thr202/Tyr204), total-p44/42, phospho-AKT (Ser473), total AKT and β-actin according to the manufacturer`s protocol (all from Cell Signalling Technology, Frankfurt am Main, Germany).

7.2.4.3 Detection of DUSP expression in AvCystatin-MΦ

For *in vitro* detection of DUSPs, peritoneal macrophages of BALB/c mice were stimulated with 0.5 µM AvCystatin or control treatments (denatured AvCystatin or SNAP-tag). After various timepoints, cells were washed with cold Dulbecco`s PBS and treated with lysis buffer for RNA extraction (Analytikjena, Jena, Germany). Furthermore, DUSP-1-deficient and wildtype mice (both C57BL/6) were intraperitoneally injected with 20 µg AvCystatin or denatured AvCystatin. Macrophages were purified from the PEC population and treated with lysis buffer for RNA extraction (Analytikjena, Jena, Germany). RNA was isolated from cell lysates, transversly transcribed into cDNA and used for quantitative real-time PCR to analyse the expression of DUSP-1, DUSP-2, DUSP-3, DUSP-5, DUSP-6 and DUSP-10. To determine DUSP-1 phosphorylation in naïve BALB/c mice or MAPK phosphorylation in DUSP-1-deficient mice and wildtype littermates, whole cell extracts of *in vitro*-stimulated macrophages were analysed by western blotting with primary antibodies against phospho-DUSP-1 (Ser359), DUSP-1, phospho-p38 (Thr180/Tyr182), phospho-p44/42 (Thr202/Tyr204) and β-actin.

7.2.5 Functional and molecular characterization of AvCystatin-MΦ

7.2.5.1 Cell purification and adoptive cell transfer

Mice were intreaperitoneally treated with 20 µg AvCystatin or control applications. PEC were isolated after 18-20 hours and either used directly for cell transfer ($3x10^6$ cells/mouse) or purified for macrophages and B cells. Purification of B cells was performed by incubating PEC with anti-CD19 MicroBeads and subsequent magnetic cell separation by autoMACS according to the manufacturer`s protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Macrophages were purified by incubating PEC with biotinylated antibodies anti-CD3ε (clone 145-2C11; Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD19 (clone eBio1D3; eBioscience, Frankfurt am Main, Germany), anti-CD11c (clone N418; eBioscience, Frankfurt am Main, Germany), anti-Gr1 (clone RB6-8C5; DRFZ, Berlin, Germany) and anti-Dx5 (clone Dx5; eBioscience Frankfurt am Main, Germany). Addition of 40 µg/ml anti-mouse FcγR (clone 2.4G2; DRFZ, Berlin, Germany) was used to avoid non-specific binding. Cells were washed with Dulbecco`s PBS supplemented with 0.2% BSA and 2 mM EDTA and incubated with anti-Biotin MicroBeads as described in the manufacturer`s protocol (Miltenyi Biotec,

Bergisch Gladbach, Germany). After incubation, magnetic cell separation was performed by using autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of macrophages (> 95 %) or B cells (90-95%) was determined by flow cytometry for the expression of F4/80, CD11b or CD19. $2x10^6$ purified macrophages or B cells were resuspended in 0.2 ml PBS and intravenously transferred into mice one day after access to DSS (model of DSS-induced colitis) or on day 24 into ovalbumin-sensitized mice prior to intranasal provocation with ovalbumin. To examine macrophage survival in recipient animals, purified macrophages were labelled with 5 µM CFSE (Sigma-Aldrich, Steinheim, Germany) for 8 minutes at room temperature. Labeling was stopped by adding 100 µl/ml FCS (Biochrom, Berlin, Germany). Cells were washed twice with Dulbecco`s PBS and adoptively transferred into recipient mice. Seven days after cell transfer, BAL-fluid, lung tissue, splenocytes and peribronchial lymph nodes were analysed for CFSE-labelled macrophages by LSRFortessa flow cytometer (BD Biosystems, Heidelberg, Germany) and FlowJo Software version 8.8.7 (Tree Star, Ashland, USA).

7.2.5.2 Model of ovalbumin-induced airway hyperreactivity

Induction of airway inflammation. Female BALB/c mice were sensitized by intraperitoneal injections on day 0 and 14 with 20 µg ovalbumin from chicken egg (Sigma-Aldrich, Steinheim, Germany) emulsified in 2 mg Imject Alum adjuvant (ThermoFisher Scientific, Schwerte, Germany) in 200 µl Dulbecco`s PBS (PAA Laboratories, Cölbe, Germany). Airway challenge was performed through intranasal application of 50 µg ovalbumin per animal on day 28 and 29. Naïve control mice were sensitized with PBS in Imject Alum adjuvant and challenged with PBS instead of ovalbumin. On day 31, blood samples were collected for antibody analysis and animals thereafter sacrificed with isoflurane (Baxter, Munich, Germany). Lungs were flushed to determine cell numbers and cytokine quantities in the BAL-fluid and afterwards fixed with 3.5% paraformaldehyde (Carl Roth, Karlsruhe, Germany) for histological analysis. In parallel, cryoconservation of lung tissue was performed and samples stored in a -80°C freezer. Moreover, the spleen was removed to prepare splenocytes for cell culture experiments.

Bronchoalveolar lavage and histological analysis of lung sections. The trachea of sacrificed mice was exposed and flushed twice with 800 µl protease inhibitor cocktail (1 complete Mini inhibitor cocktail tablet from Roche, Mannheim, Germany dissolved in 10 ml Dulbecco`s PBS) through a tracheal drain tube (BD Bioscienses, Heidelberg, Germany). The weight of both lavages was measured and the supernatant of the first lavage stored at -20 °C for cytokine detection. After a centrifugation step (2000 rpm, 10 min, 4°C), the cell pellets of

both lavages were pooled in a total volume of 1 ml Dulbecco`s PBS and counted. Cytospin slides were prepared from 100 µl BAL fluid by using the cytospin centrifuge cellspin I (Tharmac, Waldsolms, Germany), stained with the Hemacolor® staining set for microscopy (Merck, Darmstadt, Germany) and analysed for different cell types in a blinded fashion. Lungs were fixed in 3.5% paraformaldehyde for histological analysis and embedded into paraffin. PAS and HE staining was performed for preparations of 5 µm slices to determine mucus production and inflammatory cell infiltration. Stained slices were analysed through light microscopy with 200-fold magnification (Leica DFC480 camera and Leica Application Suite Software version 2.8.1 from Leica, Solms, Germany). Staining and analysis of lung sections was performed by Christoph Loddenkemper and Anja Kühl, Research Center ImmunoSciences, Immunopathology, Charité Hospital, Berlin, Germany.

Chemokine detection in lung homogenates. Lung tissue was homogenized by using FastPrep-24 lysing matrix tubes D and the FastPrep-24 homogenizer according to the manufacturer`s guidelines (both MP Biomedicals, Heidelberg, Germany). RNA was extracted from lung homogenates, transcribed into cDNA and quantitative real-time PCR performed for specific chemokines and chemokine receptors.

Splenocyte preparation and stimulation. The spleen was isolated from euthanized mice under sterile conditions and mashed through a 70 µm nylon cell strainer (BD Biosciences, Heidelberg, Germany) in Dulbecco`s PBS containing 0.2% BSA. Cells were centrifuged (1200 rpm, 4°C, 10 min) and resuspended in 5-8 ml erythrocyte lysis buffer (0.02 M $KHCO_3$, 0.155 M NH_4Cl, 0.1 mM EDTA). After incubation on ice for 5 minutes, cells were washed, counted and adjusted to the favoured cell concentration in RPMI supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin (cRPMI). $3x10^5$ splenocytes were seeded in 96 well plates (Corning Costar, Bodenheim, Germany) and stimulated with 50 µg/ml ovalbumin, 2.5 µg/ml concavalin A (both GE Healthcare, Munich, Germany) or 10 µg/ml AvCystatin. After 96 hours, culture supernatants were collected and stored at -20°C for cytokine detection.

Cytokine- and sublass-specific ELISA. Cytokine levels were assessed by sandwich ELISA for IFN-γ, IL-4, IL-5, IL-13 (all from eBioscience, Frankfurt am Main, Germany) and IL-10 (BD Biosciences, Heidelberg, Germany) in the BAL-fluid and splenocyte culture supernatants. For the detection of ovalbumin-specific IgE, 96 well flat bottom ELISA plates (ThermoFisher Scientific, Schwerte, Germany) were coated with 10 µg/ml ovalbumin at 4°C overnight and unspecific binding blocked by 3% BSA (AppliChem, Darmstadt, Germany) in PBS for one hour. After incubation with mouse serum for two hours, anti-mouse IgE-HRP (clone

MCA419P; Serotec, Düsseldorf, Germany) was used as detection antibody and specific binding visualized by application of the HRP-substrate TMB (Sigma-Aldrich, Steinheim, Germany). Ovalbumin-specific IgG1 and IgG2 were measured in an analogical manner whereas alkaline phosphatase-conjugated anti-mouse IgG1 and IgG2a (both clone Polyclonal; Rockland Immunochemicals, Gilbertsville, USA) were used as detection antibodies and nitrophenylphosphat (Sigma-Aldrich, Steinheim, Germany) as substrate. To calculate antibody amounts, purchasable anti-mouse IgE, κ- (clone C38-2), IgG1, κ- (clone MOPC-31C) and IgG2a, κ- (clone G155-178) standards were used (all from BD Biosciences, Heidelberg, Germany). To determine total IgE levels, 96 well flat bottom plates were coated with 4 µg/ml anti-mouse IgE capture antibody (clone R35-72; BD Biosciences, Heidelberg, Germany) at 4°C overnight and further treated as described for ovalbumin-specific IgE.

7.2.5.3 Model of DSS-induced colitis

Colitis was induced by providing 3% DSS (MP Biomedicals, Heidelberg, Germany, Lot No. 6683K) dissolved in drinking water to female CL57/BL6 mice over a total time period of 8 days. $3x10^6$ AvCystatin- or control-treated macrophages were intravenously administered into mice one day after access to DSS. Changes in body weight were determined during day 0 and day 8. After dissection on day 8, the colon length of each animal was measured and parts of distal colon fixed in 3.5% paraformaldehyde (Carl Roth, Karlsruhe, Germany) for histological analysis. Stained colon sections were analysed by light microscopy with 100-fold magnification (Leica DFC480 camera and Leica Application Suite Software version 2.8.1 from Leica, Solms, Germany). Colon tissue was further treated with HBBS containing 1% BSA (AppliChem, Darmstadt, Germany) and 2 mM EDTA (AppliChem, Darmstadt, Germany) to remove the intestinal epithelial layer and digested with collagenase VIII (Sigma Aldrich, Steinheim, Germany) for one hour at 37°C in a water bath. Separation of lamina propria mononuclear cells was performed by Percoll gradient (GE Healtcare, Munich, Germany). Lamina propria cells were stained with PE-conjugated anti-mouse SiglecF (clone E50-2440; BD Biosciences, Heidelberg, Germany) and analysed by flow cytometry (LSRFortessa, BD Biosystems, Heidelberg, Germany) and FlowJo software version 8.8.7 (Tree Star, Ashland, USA). Quantification of inflammatory cells was performed for $F4/80^+$, $MPO7^+$, $CD3^+$ and $B220^+$ cells by counting the respective cells in 10 high power fields of stained colon slices. Staining and analysis of distal colon sections was performed by Anja Kühl, Research Center ImmunoSciences, Immunopathology, Charité Hospital Berlin, Germany. Histopathology was determined by using the following scoring scheme:

Scoring = infiltration + tissue damage (0-6)

inflammation:
0: no inflammation
1: increased number of inflammatory cells in lamina propria
2: inflammatory cells extending into submucosa
3: transmural inflammatory infiltrates

tissue damage:
0: no mucosal damage
1: discrete epithelial lesion
2: erosion or focal ulceration
3: severe mucosal damage with extended ulcerations extending into bowel wall

7.2.5.4 Macrophage characterization

Transcriptional profiling. For the characterization of *in vitro*-stimulated macrophages, PEC were isolated from BALB/c mice, seeded in 24 well plastic tissue-culture dishes (Corning Star, Bodenheim, Germany) and incubated at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin (cDMEM). After 4 hours, non-adhering cells were removed by washing with pre-warmed cDMEM. Remaining macrophages were cultured over night in cDMEM and stimulated on the following day with either 0.5 µM AvCystatin or control treatments. Macrophages were washed with Dulbecco`s PBS after indicated timepoints and frozen at -80°C in lysis buffer for RNA extraction (Analytikjena, Jena, Germany). For the characterization of *in vivo*-stimulated cells, macrophages were purified from isolated PEC of AvCystatin- or control-treated animals 18-20 hours after treatment as described earlier. Purified macrophages were treated with lysis buffer for RNA extraction (Analytikjena, Jena, Germany) and frozen at -80°C.

Surface marker profile. Surface marker expression was determined for F4/80$^+$CD11b$^+$ macrophages 18-20 hours after intraperitoneal treatment with AvCystatin or controls by using anti-MHC-II-FITC (clone M5/114.15.2; eBioscience, Frankfurt am Main, Germany), anti-CD274-PE (clone MIH5; BD Biosciences, Heidelberg, Germany), anti-CD273-PE (clone TY25; BD Biosciences, Heidelberg, Germany), anti-CD40-PE (clone 1C10; eBioscience, Frankfurt am Main, Germany), anti-CD86-Biotin (clone GL1; eBioscience, Frankfurt am Main, Germany), streptavidin-PECy7 (clone Streptavidin; BD Biosciences, Heidelberg, Germany), anti-CD16/32-APC (clone 2.4G2; DRFZ, Berlin, Germany), anti-CD80-PE (clone 16-10A1; eBioscience, Frankfurt am Main, Germany) and anti-ICAM-1-APC (clone KAT1; DRFZ,

Berlin, Germany). Non-specific binding was prevented by adding 20 µg/ml anti-mouse FcγR (clone 2.4G2; DRZF, Berlin, Germany). After antibody binding, cells were washed with FACS buffer and flow cytometry performed by using LSRFortessa flow cytometer (BD Biosciences, Heidelberg, Germany) and FlowJo Software version 8.8.7 (Tree Star, Ashland, USA).

7.2.5.5 *In vivo* expression of IL-10 in $CD4^+$ T cells

$CD4^+$ T cells were purified from the spleen of ovalbumin-sensitized and -challenged mice seven days after adoptive transfer of macrophages by using the $CD4^+$ T cell isolation kit according to the manufacturer`s guidelines (both from Miltenyi Biotec, Bergisch Gladbach, Germany). $1x10^6$ purified $CD4^+$ T cells were stimulated with ovalbumin (50 µg/ml) or ConA (2.5 µg/ml) for 96 hours in cRPMI. The culture supernatants were collected and frozen for IL-10 cytokine ELISA (BD Biosciences, Heidelberg, Germany). Cells were treated with lysis buffer for RNA extraction (Analytikjena, Jena, Germany). RNA was isolated from cell lysates, transcribed into cDNA and IL-10 expression determined by quantitative real-time PCR.

7.2.5.6 *In vitro* co-culture assays

Cell generation and preparation. To generate dendritic cells from BALB/c mice, the bone marrow from the femur and tibia of murine hind legs was flushed with RPMI supplemented with 100 U/ml penicillin G and 100 mg/ml streptomycin and collected in a pre-cooled sterile petri dish (Sarstedt, Nümbrecht, Germany). The marrow was dissociated by slightly pipetting up and down and cells filtered through a 70 µM nylon cell strainer (BD Biosciences, Heidelberg, Germany). After centrifugation (1200 rpm, 4°C, 10 min), erythrocyte lysis was performed for 5 minutes on ice. Cells were washed, counted, seeded in a petri dish with a density of $4x10^6$ cells in 10 ml cRPMI supplemented with 20 ng/ml murine GM-CSF (PreproTech, Hamburg, Germany) and incubated at 37°C and 5% CO_2. Three days later, 10 ml pre-warmed cRPMI containing 10 ng/ml GM-CSF was added. Dendritic cells were harvested on day seven, resuspended in fresh cRPMI and either directly used for co-culture assays or pre-treated with 1 µg/ml ovalbumine-peptide 323-339 (GenScript, Piscataway, USA). After incubation at 37°C for two hours, the remaining peptide was washed away and dendritic cells were used for co-culture experiments. Splenocytes were prepared from the spleen of euthanized DO11.10 TGN Jackson mice and ovalbumin-specific $CD4^+$ T cells purified by using the $CD4^+$ T cell isolation kit according to the manufacturer`s description (Miltenyi Biotec, Bergisch Gladbach, Germany). Purification of macrophages from AvCystatin- (20 µg/mouse) or control-treated BALB/c mice was performed as described previously.

In vitro co-culture. Ovalbumin-specific $CD4^+$ T cells were incubated with macrophages and ovalbumin-peptide-treated dendritic cells in a ratio of 5:1:1. The assays were performed in a mixed population and a transwell approach separating the macrophages from dendritic cells and T cells through a 1.0 µm filter membrane (greiner bio-one, Frickenhausen, Germany). $CD4^+$ T cells and dendritic cells were additionally incubated in the presence of fresh macrophage supernatants. Therefore, macrophages were cultured separately for 18 hours and the macrophage culture supernatant was subsequently added to $CD4^+$ T cells and dendritic cells. Culture supernatants were collected after 48 hours and analysed by ELISA for IL-10 (BD Biosciences, Heidelberg, Germany), IL-2, IFN-γ and IL-13 (all from eBioscience, Frankfurt am Main). Remaining cells were intracellularly stained for IL-10. Therefore, cells were stimulated with 1 µg/ml PMA/ionomycin (Sigma-Aldrich, Steinheim, Germany) for three hours in fresh cRPMI at 37°C and 5% CO_2. After 30 minutes, 1 µg/ml brefeldin A (Sigma-Aldrich, Steinheim, Germany) was added. Cells were washed with FACS buffer, stained with anti-CD4-PerCP-eF710 (clone RM4-5; eBioscience, Frankfurt am Main, Germany) and incubated for 15 minutes on ice. In addition, 20 µg/ml anti-mouse-FcγR (clone 2.4G2; DRFZ, Berlin, Germany) was added to prevent unspecific binding. After being washed, cells were permeabilized with the fixation/permeabilization kit from BD Biosciences, Heidelberg, Germany according to the manufacturer`s instructions, intracellularly stained with anti-IL-10-PE (clone JES5-16E3; eBiosciences, Frankfurt am Main, Germany) and analysed by LSRFortessa flow cytometer (BD Biosciences, Heidelberg, Germany) and FlowJo software version 8.8.7 (Tree Star, Ashland, USA). To evaluate an autocrine impact of IL-10 on $CD4^+$ T cell cytokine responses, splenic $CD4^+$ T cells of DO11.10 TGN Jackson mice were pre-incubated with 10 µg/ml anti-IL-10 receptor antibody (clone 1B1; DRFZ, Berlin, Germany) for one hour and finally co-cultured with AvCystatin- or control-stimulated macrophages and ovalbumin-peptide-treated dentritic cells as described previously. Levels of IL-10, IL-2, IFN-γ and IL-13 were quantified in the culture supernatants by ELISA.

Co-culture experiments using macrophage populations. Peritoneal macrophages were purified from naïve BALB/c mice and *in vitro* polarized into M1, M2a, M2b and AvCystatin-macrophages. For M1 polarization, macrophages were treated with 100 U/ml IFN-γ (PreproTech, Hamburg, Germany) and 10 ng/ml LPS (Sigma-Aldrich, Steinheim, Germany) for 18-20 hours. M2a polarization was performed by pre-treating macrophages with 20 ng/ml IL-21 for 6 hours and subsequent incubation with 20 ng/ml IL-4/IL-13 for 18-20 hours (all from PreproTech, Hamburg, Germany). For M2b polarization, macrophages were stimulated with 150 µg ovalbumin immune complexes (Acris Antibodies, San Diego, USA) and 10 ng/ml LPS (Sigma-Aldrich, Steinheim, Germany) for 18-20 hours. AvCystatin- and control-treated macrophages were induced either *in vitro* through stimulation with 0.5 µM AvCystatin or the

same amount of denatured AvCystatin or *in vivo* through intraperitoneal injection of AvCystatin or control treatments. All *in vitro* stimulations were performed in cRPMI at 37°C and 5% CO_2. Macrophages were afterwards washed with fresh cRPMI to ensure that the respective polarizing stimuli do not affect other cells in the co-culture. *In vivo*-generated AvCystatin- and control-macrophages were isolated and purified from the peritoneal cavity 18-20 hours after intraperitoneal injection. Both *in vitro*- and *in vivo*-polarized macrophages were either incubated alone for 48 hours to determine IL-10 self-production by the different populations or in combination with ovalbumin-specific $CD4^+$ T cells and ovalbumin-peptide-treated dendritic cells. IL-10 levels were measured by ELISA in the supernatants and compared between macrophage cultures and co-cultures of macrophages, $CD4^+$ T cells and dendritic cells.

Analysis of macrophage markers for the induction of $CD4^+IL\text{-}10^+$ T cells. To investigate the effect of sphingosine kinase-1 on the IL-10 induction in T cells, ovalbumin-specific $CD4^+$ T cells were pre-treated with different concentrations of the S1PR1 antagonist VPC23019 (Avanti Polar Lipids, Alabaster, USA) for one hour at 37°C and 5% CO_2 and thereafter incubated with *in vivo*-stimulated macrophages and ovalbumin-peptide-treated dendritic cells. In parallel, co-cultures of $CD4^+$ T cells and dendritic cells were supplemented with various concentrations of S1P (Calbiochem/Merck, Darmstadt, Germany). To determine a possible function of ARG-1 or programmed death ligands for the induction of IL-10 in $CD4^+$ T cells, *in vivo*-generated AvCystatin-MΦ were treated in a dose-dependent manner with the ARG-1 inhibitor BEC (Calbiochem/Merck, Darmstadt, Germany) or anti-PD-L1 and anti-PD-L2 antibodies (both from eBioscience, Frankfurt am Main, Germany) for one hour at 37°C and 5% CO_2 and subsequently cultured with $CD4^+$ T cells and dendritic cells. The activity of BEC was tested by measuring urea concentrations in the supernatants of M2a macrophage cultures by using the QuantiChrom urea assay kit according to the manufacturer`s protocol (BioAssay Systems, Hayward, USA). Both, anti-PD-L1 and anti-PD-L2 were formerly evaluated for their inhibitory potential by Terrazas et al. (2005). All co-culture experiments were performed in cRPMI.

7.2.6 Statistical analysis

Statistical analysis and graphical output was performed with GraphPad Prism Software, version 5.02 (GraphPad Software, San Diego, USA). Data is presented as mean ± SEM and was first tested with a one-way ANOVA test followed by the Bonferroni´s multiple comparison test to retrieve statistical significance between experimental groups. Differences between individual groups were tested to be significant for $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

8 ABBREVIATIONS

2-D PAGE	two-dimensional polyacrylamide gel electrophoresis
AHR	airway hyperresponsiveness
ALD-DNA	activated lymphocyte-derived DNA
AN	adriamycin nephropathy
AP	alkaline phosphatase
APC	antigen-presenting cell
ARG-1	arginase-1
As-MIF	macrophage inhibitory factor of *Anisakis simplex*
Av	Acanthocheilonema viteae
AvCystatin	17 kDa cysteine protease inhibitor of *Acanthocheilonema viteae*
AvCystatin-MΦ	AvCystatin-stimulated macrophages
BAL-fluid	bronchoalveolar lavage fluid
BEC	S-(2-boronoethyl)-L-cysteine
BFA	brefeldin A
BfR	Bundesinstitut für Risikobewertung
BMMΦ	bone marrow-derived macrophages
BSA	bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
CCL	C-type chemokine ligand
CCR	C-type chemokine receptor
CD	cluster of differentiation
CD	Crohn`s disease
CFSE	carboxyfluorescein succinimidyl ester
ConA	concanavalin A
CREB	cAMP response element-binding protein
CTLA-4	cytotoxic T-lymphocyte antigen 4
Cx43	connexin 43
DC	dendritic cell

DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
Der p1	*Dermatophagoides pteronyssinus* 1 antigen
DMSO	dimethyl sulfoxide
DNBS	dinitrobenzene sulfonic acid
DRFZ	Deutsches Rheumaforschungszentrum
DSS	dextran sodium sulfate
DUSPs	dual-specificity phosphatases
EAE	experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveoretinitis
ECP	eosinophil cationic protein
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPO	eosinophil peroxidase
ERK	extracellular signal-regulated kinase
ES	excretory-secretory
ES-62	62 kDa excretory-secretory product of *Acanthocheilonema viteae*
FACS	fluorescence-activated cell sorter
FAK	focal adhesion kinase
FasL	Fas ligand
FcR	fragment crystallizable (region) receptor
FCS	fetal calf serum
FcγR	fragment crystallizable γ receptor
FheCL1	*Fasciola hepatica* cathepsin L1
FITC	fluorescein isothiocyanate
Foxp3	forkhead box transcription factor p3
FPLC	fast protein liquid chromatography
GalNAc	N-acetyl-D-galactosamine
GM-CSF	granulocyte-macrophage colony stimulating factor
HE	hematoxylin/eosin

HES	*Heligmosomoides polygyrus* excretory-secretory antigen
HGF	hepatocyte growth factor
HVEM	herpes virus entry mediator
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
IBD	inflammatory bowel disease
IC	immune complexes
ICAM-1	intercellular adhesion molecule 1
IFN-γ MdCs	IFN-γ-stimulated monocyte-derived cells
IFN-γ	interferon gamma
Ig	immune globulin
IL	interleukin
IL-4R	IL-4 Receptor
IMPRS	International Max Planck Research School
iNOS	inducible nitric oxide synthase
IPSE	IL-4-inducing principle of *Schistosoma mansoni* eggs
IRF-1	interferon regulatory factor-1
IRI	ischemia/reperfusion injury
ISAAC	international study of asthma and allergies in childhood
JNK	C-Jun N-terminal kinase
KC	keratinocyte chemoattractant
LAL	limulus amoebocyte lysate
LIX	lipopolysaccharide-induced CXC chemokine
LNFPIII	lacto-N-fucopentaose III
LNnT	lacto-N-neotetraose
LPS	lipopolysaccharide
lyso-PS	lysophosphatidylserine
MACS	magnetic-activated cell sorting

Maf	musculoaponeurotic fibrosarcoma
MAPK	mitogen-activated protein kinases
MBL	mannan-binding lectin
MBP	major basic protein
MCP	macrophage chemoattractant protein
MGL	macrophage galactose-type lectin
MHC-II	major histocompatibility complex II
MIF	macrophage migration inhibitory factor
MIP-2	macrophage inflammatory protein 2
MLN	mesenteric lymph nodes
MOG	myelin oligodendrocyte glycoprotein
MPO	myeloperoxidase
MPS	mononuclear phagocyte system
mTOR	mammalian target of rapamycin
MΦ	macrophage
NF-kappaB	nuclear factor-kappa B
NK cells	natural killer cells
NO	nitric oxide
OVA	ovalbumin
OVA-IC	ovalbumin immune complexes
OvGalBP	*Onchocerca volvulus* beta galactoside binding protein
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PAS	periodic acid-Schiff
PAS-1	protein from *Ascaris suum* 1
PBLN	peribronchial lymph nodes
PCNA	proliferation cellular nuclear antigen
PCR	polymerase chain reaction
PD-L	programmed death-ligand

PE	phycoerythrin
PEC	peritoneal excudate cells
PI3K	phosphatidylinositol-3-kinase
PMA	phorbol-12-myristate-acetate
PPAR-γ	peroxisome proliferator-activated receptor gamma
PPIA	peptidylprolyl isomerase A
PRRs	pattern recognition receptors
PTEN	phosphatase and tensin homologue
RANTES	regulated upon activation, normal T cell expressed, and secreted
RDLN	renal draining lymph nodes
RELM-α	resistin-like molecule alpha
ROS	reactive oxygen species
S1P	sphingosine-1-phosphate
S1PR	sphingosine-1-phosphate receptor
SAP	serum amyloid P component
SCF	stem cell factor
SCID	severe combined immunodeficient
SDS	sodium-dodecyl-sulfate
SEA	soluble egg antigen
sFasL	soluble Fas ligand
sLIGHT	soluble LIGHT
SmCB1	*Schistosoma mansoni* cathepsin B1 protease
Sp1	specificity protein 1
SPARC	secreted protein acidic and rich in cysteine
SPHK-1	sphingosine kinase-1
Src	sarcoma
STAT	signal transducer and activator of transcription
TAICs	transplant acceptance-inducing cells
TAM	tumor-associated macrophages

TES	*Toxocara canis* excretory-secretory antigen
TGF-β	transforming growth factor beta
TGH-2	TGF-ß homologue 2 of *Brugia malayi*
Th1	T helper cell 1
Th2	T helper cell 2
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
TNFSF14	tumor necrosis factor ligand superfamily member 14
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
UC	ulcerative colitis
UUO	unilateral ureteral obstruction
VCAM-1	vascular cell adhesion molecule 1
wt	wildtype
ZIBI	Zentrum für Infektionsbiologie und Immunität
z-Phe-Ala-fmk	benzyloxycarbonyl-Phe-Ala-fluoromethylketone

9 LIST OF ILLUSTRATIONS

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11 PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

11.1 Publications and patent applications

Thomas Ziegler, Christian Klotz, Sebastian Rausch, Matthew R. Hepworth, Svenja Steinfelder, Emilia Danilowicz-Luebert, Anja Kühl, Ute Hoffmann, Paul Burda, Alf Hamann, Richard Lucius and Susanne Hartmann (2011). A regulatory macrophage population induced by a helminth immunomodulator instructs $CD4^+$ T cells to produce IL-10 and protects against unrelated inflammation (in preparation).

Thomas Ziegler, Christian Klotz, Sebastian Rausch, Noelle O`Regan, Svenja Steinfelder, Richard Lucius, Susanne Hartmann, (2012). Regulatory Macrophages and Their Uses. European patent application EP2012/167693.

Christian Klotz, Thomas Ziegler, Ana Sofia Figueiredo, Sebastian Rausch, Matthew R. Hepworth, Nadja Obsivac, Christine Sers, Peter Hammerstein, Richard Lucius and Susanne Hartmann (2011). A helminth immunomodulator exploits host signalling events to regulate cytokine production in macrophages. PLoS Pathogens 7(1): e1001248.

Christian Klotz, Thomas Ziegler, Emilia Danilowicz-Luebert and Susanne Hartmann (2010). Cystatins of parasitic organisms. Adv Exp Med Biol. 712: 208-21.

11.2 Contributions at scientific meetings

Oral presentation: Thomas Ziegler. Molecular and functional characteristics of a helminth immunomodulator-induced suppressive macrophage population. Tolerance Club, DRFZ/MPIIB, Berlin, Germany, November 2011.

Poster presentation: Thomas Ziegler, Christian Klotz, Richard Lucius, Susanne Hartmann. Nematode-elicited macrophages ameliorate asthma upon adoptive transfer. ZIBI Graduate School Retreat, Potsdam, Germany, February 2011.

Oral presentation: Thomas Ziegler. Nematode-induced macrophages mediate protection against allergic airway inflammation. Annual ZIBI Graduate School presentation, Humboldt University at Berlin, Berlin, Germany, December 2010.

Oral presentation: Thomas Ziegler. Characterization of regulatory macrophages. SFB650 Retreat, DRFZ/MPIIB, Berlin, Germany, November 2010.

Poster presentation: Thomas Ziegler, Susanne Hartmann. Induction of regulatory macrophages by a helminth immunomodulator. IMPRS Evaluation, MPIIB, Berlin, Germany, April 2010.

Oral presentation: Thomas Ziegler. Induction of regulatory macrophages by a helminth immunomodulator. Joint Meeting of the German Societies of Parasitology and Protozoology, Heinrich-Heine University, Duesseldorf, Germany, March 2010.

Oral presentation: Thomas Ziegler. Characterization of myeloid suppressor cells upon stimulation with the filarial immunomodulator AvCystatin. Annual ZIBI Graduate School presentation, Humboldt University at Berlin, Berlin, Germany, November 2009.

Poster presentation: Thomas Ziegler, Christian Klotz, Richard Lucius, Susanne Hartmann. Characterization of suppressive macrophages upon stimulation with the nematode immunomodulator AvCystatin. 13th Annual Woods Hole Immunoparasitology Meeting, Woods Hole, USA, April 2009.

Oral presentation: Thomas Ziegler. Cross-talk among receptors/pathways in AvCystatin-mediated signalling. Annual ZIBI Graduate School presentation, Humboldt University at Berlin, Berlin, Germany, February 2009.

Poster presentation: Thomas Ziegler, Christian Klotz, Richard Lucius, Susanne Hartmann. Characterization of myeloid suppressor cells upon stimulation with helminth immunomodulators. DFG/GRAKO-1121 Evaluation, Humboldt University at Berlin, Berlin, Germany, January 2009.

Poster presentation: Thomas Ziegler, Christian Klotz, Susanne Hartmann. Characterization of myeloid suppressor cells upon stimulation with helminth immunomodulators. ZIBI Graduate School Retreat, Gut Frohberg, Germany, December 2008.

12 STATUTORY DECLARATION

I hereby declare that the thesis has been written by myself without any external unauthorised help and that I have not used other than the declared sources.

Berlin, 12th of June 2012

Thomas Ziegler

13 EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, die vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt zu haben.

Berlin, den 12. Juni 2012

Thomas Ziegler